ELISA
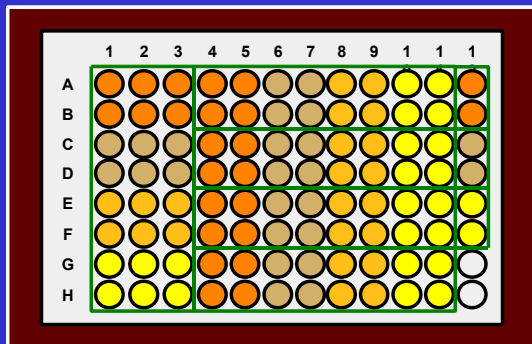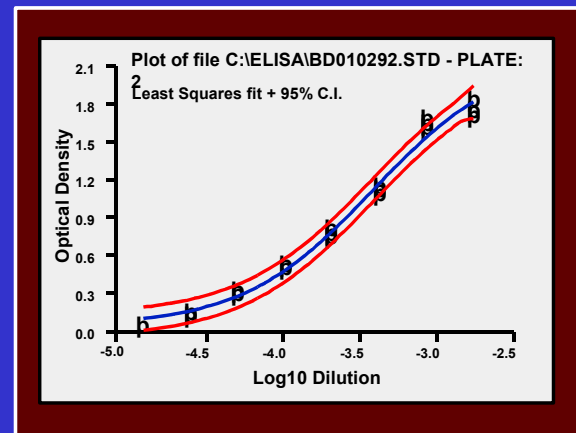## *for Windows*

User's Manual

- Customized ELISA plate formats

- Robust regression

- Iteratively reweighted least squares

- Outlier detection

- Antibody quantitation

CDC

Program **ELISA** *for Windows*

Version 2.00

User's Manual

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Table of Contents

List of Figures

INTRODUCTION

        ELISA *for Windows* is a collection of public domain programs prepared for general release to those individuals who typically perform assays using a 96-well ELISA plate. This program is flexible and applicable to a wide body of researchers and analysis protocols.

        We are pleased to distribute the routines to anyone who requests them with the proviso that each investigator will need to spend some time learning the functions of each module to benefit from the whole collection of programs. The authors also request that they be cited as the source for this work when these techniques are incorporated into new or existing software or used in the preparation of published reports. A suggested citation for **ELISA** *for Windows* appears at the bottom of the title page of this manual.

        The programs are complex and contain many checks to monitor for user errors when responding to screen prompts. However, it is not feasible to make these error checks exhaustive, and it is possible to lock up the program by entering illogical responses to screen prompts or by selecting various options in an incorrect order. Users may guard against these eventualities by exercising care in forming their analysis strategies and responses to various inquiries.

        This documentation was prepared as carefully as possible and, hopefully, is complete to the extent that most users will be able to install and use **ELISA** *for Windows* with minimal complications. We would appreciate being notified of any additions or modifications which could be made to this manual to clarify the program's operation.

        This documentation was intentionally written to be verbose and, at times, somewhat repetitive. We have attempted to anticipate all problems and questions involved with running the programs. The repetitive nature of this document is meant to facilitate running the different modules without having to leaf back and forth through the manual looking for pertinent information related to the task at hand. It should be noted that we are not able to test every possible combination of options and prompts which the program offers. We also cannot foresee all possible computer configurations, hard disk subdirectory

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structures, etc. which may cause **ELISA** *for Windows* to perform erratically. If any inconsistencies do occur, we would appreciate being notified so that we may be able to rectify any problems. In the long run, we believe that in its present implementation, **ELISA** *for Windows* is extremely stable. We would not distribute it otherwise.

Questions related to program execution should be directed to one of the members of the Respiratory Diseases Immunology Section, Respiratory Diseases Branch, DBMD/NCID/CDC. Questions regarding the theoretical background of the techniques involved (e.g., the iteratively reweighted robust/least squares fit) as well as program 'bugs' should be directed to Brian D. Plikaytis, Chief, Biostatistics Section, Biostatistics and Information Management Branch/DBMD/NCID/CDC.

What's New

The main improvement between the present version of **ELISA** and previous versions is the addition of a mechanism to pre-identify and censor optical densities prior to estimating antibody concentrations in Module 6 - Calculate Concentrations. With this feature, an analysis strategy may be developed that will automatically eliminate untrustworthy optical densities from mean antibody concentrations for serum specimens.

With this version of **ELISA** *for Windows*, standard curve parameters **must** be estimated (Module 4) prior to data analysis in Module 6 - Calculate Concentrations.

The Batch Mode Processing module has been eliminated.

Getting Started

Installation

ELISA *for Windows* is a 16-bit application and will run under Windows 3.x or Windows 95.

For Windows 3.x - start a normal Windows session. From the Windows Program Manager, select File from the menu bar and click on Run. The Run dialog box will appear. Place disk 1 into the A: or B: drive. Type A:SETUP.EXE or B:SETUP.EXE in the Command Line box and click on OK. Windows 3.x will proceed to install the software. When the installation is complete, activate the **ELISA** program icon by single clicking on it with the *left* mouse button. Now open the File Properties dialog window by clicking on File in the Program Manager and then selecting Properties. Enter the Command Line box where the statement to execute **ELISA** is located. Add a space and then enter the subdirectory where the program is stored AFTER the execution statement. Thus, if the program is stored in a subdirectory called ELISAWIN, the text in the Command Line box should read: C:\ELISAWIN\ELISA.EXE C:\ELISAWIN. Be careful not to put a trailing '\' after the subdirectory name as the program will do this automatically when it is required.

For Windows 95 - start the normal Windows session. Click on Start located on the task bar. Click on Run which will produce the Run dialog box. Place disk 1 into the A: or B: drive. Type A:SETUP.EXE or B:SETUP.EXE in the Open box and click on OK. Windows 95 will proceed to install the software. When the installation is complete, activate the program environment pop-up menu box by single clicking on the **ELISA** icon with the *right* mouse button. Click on Properties and then select the Shortcut folder. Enter the Target box where the statement to execute **ELISA** is located. Add a space and then enter the subdirectory where the program is stored AFTER the execution statement. Thus, if the program is stored in a subdirectory called ELISAWIN, the text in the Target box should read: C:\ELISAWIN\ELISA.EXE C:\ELISAWIN. Be careful not to put a trailing '\' after the subdirectory name as the program will do this automatically when it is required. Note that **ELISA** *for Windows* is not a 32 bit Windows 95 application and will not support the lengthy filenames that Windows 95 will accommodate.

ELISA *for Windows* will run from a network environment if the program is assigned a shared attribute. On networks running under the Novell® Netware® operating system, this is done using the 'FLAG' command. If **ELISA** *for Windows* is loaded onto a local hard disk drive, it is advisable to reserve a subdirectory specifically

for the program files. Data processed by the program may then be relegated to one or more separate subdirectories. This practice will ease future problems related to file management as **ELISA** *for Windows* will generate several files during processing. It is not necessary to assign all data to the same subdirectory. Users are free to create different disk directories and subdirectories which correspond to different projects. It is an easy matter to select the appropriate subdirectory for file recall and storage in the file selection dialog windows.

Standard Curve Formation

ELISA *for Windows* uses several techniques for estimating standard or calibration curve parameters. Users may employ a parametric model, the four-parameter logistic-log function to form standard curves. In this case, the user may exercise several fitting options when forming standard curves. An iteratively unweighted least squares method and a robust iteratively reweighted least squares technique may be employed. Users may also select between two estimation algorithms, Taylor series-linearization, and Marquardt's Compromise. These options are described in much more detail in the parameter estimation section of this documentation. Alternatively, a spline fit may be used for those instances where the parametric curves cannot adequately describe the standards. The spline may be selected in the standard curve plotting module or the antibody concentration calculation module. The standard curve line described by the spline fit is programmed to pass through the median value of the replicate optical densities at each dilution along the curve.

Standard Curve Plot Printout

ELISA *for Windows* will display fully annotated logistic-log and spline fit plots of the standards with the best fitting line drawn through the data points. A hard copy of the standard curve plots may be obtained using a Windows printer driver. For the present, this facility only works if the number of colors set for the Windows environment is less than or equal to 256. Depending on the Windows implementation, this may be set using a monitor program from the Control Panel or a special utility supplied by the manufacturer of the video board installed in the given computer.

Module Output

Numeric output from the various modules is formatted as concisely as possible while retaining the maximum degree of information. In almost all cases, procedure

output is designed to span a fixed 80 column line so that results may be viewed on-screen as well as directed to a printer. While designing the output format for each of the modules, we endeavored to allocate enough spaces for numeric results which would accommodate most any data processed by the program. If numeric results exceed available space unpredictable results may occur (e.g., 0's reported instead of real results). This should not be an issue with normal data sets. If it does prove to be a problem, please inform us. Module output is directed to a windowing screen with both vertical and horizontal scroll bars along the right and bottom edges, respectively, of the window. Users may scroll through the output using these scroll bars. Additionally, some windows permit users to position the cursor anywhere within the frame and enter comments from the keyboard. These functions will be described more fully in the pertinent sections of this documentation.

All output may be directed to a file for later retrieval and printing using any word processing program. The output is stored in standard ASCII text format.

Dialog Windows

Dialog windows are used throughout **ELISA** *for Windows* and serve as a mechanism for users to input various parameters and select options for module execution. In general, dialog windows are composed of three basic constructs for user input, check boxes, option buttons, and text boxes and appear as:

```
[X]     Check box
(°)     Option button
[ ]     Text box`
```

Check boxes are used to turn any number of user options on [X] and off [ ]. By clicking on the check box with the left mouse button, users are able to select/deselect check box items.

Option buttons are arranged in groups and are mutually exclusive. That is, a user may select only one option from a group of options. Also, one option button in a group must be selected. Option buttons are activated (°) by clicking on them with the left mouse button.

Text boxes are used for free-field text entry (e.g., titles for reports) or to enter numbers which are used in subsequent calculations (e.g., initial parameter estimates for standard curve estimation). When numbers are required, **ELISA** *for Windows* will restrict text entry to just numeric data, beeping if a letter or other nonnumeric character is entered. Text boxes are accessed by clicking anywhere between the square brackets. Data or text are then entered from the keyboard.

ELISA *for Windows* will restrict the selection of some options based on previous user input. An example of this feature may be found in the standard curve plotting module where users may choose from three possible fitting techniques (robust, un-weighted least squares, and spline fits). In this circumstance, **ELISA** *for Windows* will only allow users to select the spline fit or any other fit chosen in the parameter estimation module for the given standards data file. That is, if the user selected just the robust fit during parameter estimation, he/she will only have access to the spline and robust fit options in the plotting module. The unweighted least squares option will be suppressed.

File Structures

Raw Data

Raw data are captured using any one of a variety of commercial (CROSSTALK, PROCOMM, etc.) or public domain communication software packages by means of a standard RS-232 link between a desktop computer and the ELISA reader used in the lab. We have installed pre-processing routines to translate output captured from a variety of readers to a uniform format which the remaining modules of the program may access. As collaborations with outside laboratories expand, additional conversion options will be added (see Module 1 for details). Note that **ELISA** *for Windows* will **not** collect raw data from these instruments. It is expected that this operation be performed prior to entering **ELISA** *for Windows*.

If the user employs a different reader, **ELISA** *for Windows* may still analyze the data as long as the raw data conform to the uniform format which **ELISA** *for Windows* expects to process. Figure 1 outlines the format for the raw data (.DAT) file required by **ELISA** *for Windows*.

Raw data files are named with a .DAT extension such as *file*.DAT, where *file* is the root name assigned by the user. The root file name may be any valid name recognized by DOS. The .DAT extension is not required for program execution but will facilitate certain operations in different program modules. It is strongly recommended that users adhere to this convention. One or more comment lines may be placed at the beginning of each plate in the manner displayed in Figure 1. These comment lines are optional. If they are included, the word **COMMENT** must be capitalized, start in column 1 and be followed by a colon (**:**). **ELISA** *for Windows* detects the end of the comment lines and the beginning of the data by the absence of the character string **COMMENT**. The data portion is arranged in the standard 8 row, 12 col-

umn ELISA plate format. Additional plates may be chained together so that a .DAT file may contain data from several plates. Comment lines are not required as **ELISA** *for Windows* simply counts each 8 rows of numbers as one plate. If comment lines are present then the third module, Abstract Data, will place these in a .LBL file. This file will later be used to annotate output from module 4, Parameter Estimation and module 6, Calculate Concentrations.

When **ELISA** *for Windows* creates .DAT files using the unprocessed data directly captured from any ELISA reader, the program translates all negative and 0.0 optical density measurements to 0.001. When users create the .DAT files, all absorbance values must be greater than or equal to 0.001. This is a requirement for the successful execution of many of the subsequent modules in the program. Since the foundation of **ELISA** *for Windows* rests on the 4 parameter logistic-log fit, the log values of the optical densities are generated in several instances. The logs of negative numbers and 0.0 are undefined. If a .DAT file contains these types of data, the program will eventually end with an invalid function call diagnostic. **ELISA** *for Windows* translates Dynatech MR5000/700 ERROR, UNDER, and BLANK codes to 0.001; OVER and any reading greater than 3.0 are translated to 3.0. When creating .DAT files, there, obviously, should not be any nonnumeric codes for absorbance values in the 8 by 12 matrix of ODs. While the maximum value is set to 3.0, this may be altered by the user through modification of the .DAT file in question.

File and Subdirectory Naming Conventions

ELISA *for Windows* creates several files some of which are transitory in nature. This is to say they are rewritten with new information each time the program is executed. Some of these files are meant for internal use by the program and are of

```
COMMENT:This is comment line 1
COMMENT:This is comment line 2
  1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234
  1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234
  1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234
  1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234
  1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234
  1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234
  1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234
  1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234
repeat for second plate.
repeat for third plate.
.
.  Include as many plates as desired.
.
```

Figure 1. Uniform format for .DAT file required by program **ELISA** *for Windows*.

little interest to the user other than to recognize their existence. Other files contain some forms of output which are generated automatically each time certain modules within **ELISA** *for Windows* are executed.

A second form of file with defined extensions is created during the data processing and analysis stages of **ELISA** *for Windows*. In this situation, the user is prompted for a root file name (name without an extension), and the program will automatically generate the necessary formatted file with a defined extension. Figure 2 outlines these different types of files. Each file or file type will be discussed in the appropriate section of this documentation. Note that some of these files will not be created until **after** the first execution of **ELISA** *for Windows*. That is, not all of these files will be included on the distribution disk containing the program.

Each of the different data file types, *file*.DAT, *file*.STD, *file*.UNK, *file*.QC, *file*.LBL, and *file*.CFT, where *file* is a joint file name created by the user and **ELISA** *for Windows*, **must** reside in the same subdirectory of the local hard disk drive or on the same diskette. These files are used in a linked fashion during data processing and analysis and are treated as a unified group. If the file group is split into two or more subdirectories, **ELISA** *for Windows* will not be able to locate all of them and the program will continue with unpredictable results.

Data and program files should not be stored in subdirectory with names that include an extension. The period (.) in a subdirectory name that includes an extension will adversely affect the parts of the program that automatically add predefined extensions to root file names. This will ultimately lead to a program execution error.

.DAT	Raw data file.
.STD	Standards data file.
.UNK	Unknowns data file.
.QC	Quality control samples file.
.LBL	Plate label file with comments from the .DAT file (If there are any).
.CFT	Coefficient file generated from Module 4 -Parameter Estimation.
.TPL	Template file details abstraction protocol for standards, unknowns and quality control samples.
.FIT	Report file from Module 4 - Parameter Estimation.
.RPT	Expanded report file from module 6 - Calculate Concentrations.
.SUM	Summary report file from Module 6 - Calculate Concentrations.
TEMPFILE	Scratch file used in the parameter estimation and concentration calculation modules.

Figure 2. File types used by program **ELISA** *for Windows*.

File Types

All files created by **ELISA** *for Windows* are ASCII text files which may be viewed using any DOS or Windows text editor. These files may additionally be used as data for input into other programs. The one exception to this is the coefficient files with the .CFT extension. The coefficient files are so-called 'random-access' files and are stored in a compressed or packed format. These files may only be read reliably by **ELISA** *for Windows*.

File Selections

All file choices in **ELISA** *for Windows* are made using file selection dialog windows. A typical dialog window for template file selection is displayed in Figure 3. Users may maneuver through the various parts of this dialog window with a variety of pointing devices (e.g., mouse, trackball, etc.). What follows is a brief description of how to select files using this dialog window. However, users will probably benefit more from experimenting and viewing the results of their own actions. Once a basic system for navigation and option selection is learned, it may be applied to all dialog windows in **ELISA** *for Windows*.

Note that in this example the File Name box is initialized with a .TPL extension (the extension assigned to template files). All files in the current subdirectory with this extension are displayed in the file list box and are arranged in alphabetical order. This extension will change, depending on the program module (e.g., .STD in the parameter estimation module, etc.). When the dialog window is opened, the cursor is initially
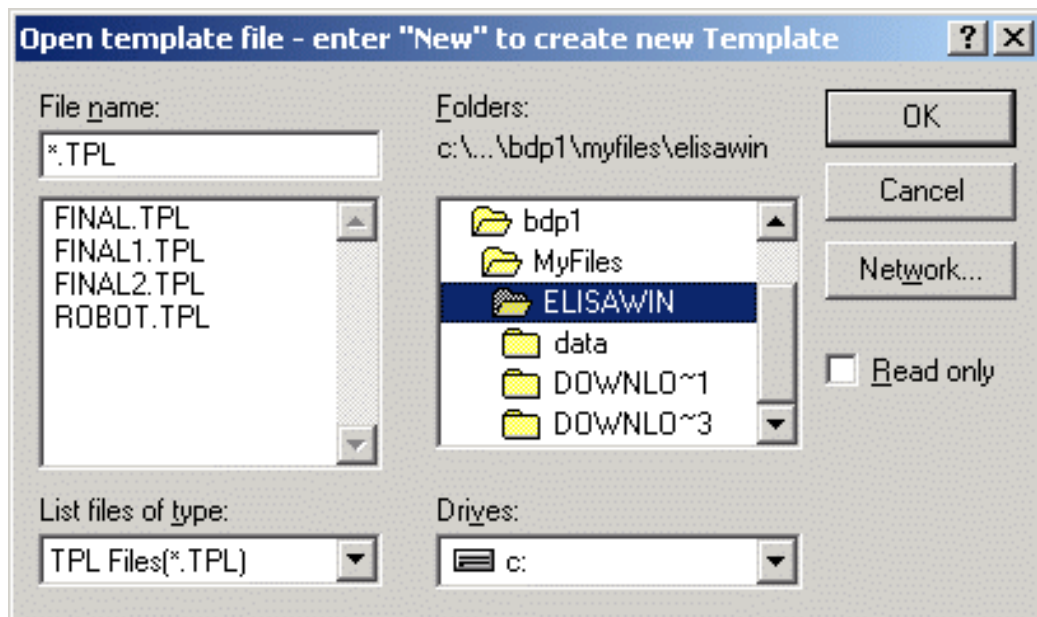

Figure 3. Sample file selection menu.

positioned in the File Name box. Alternatively, pressing Alt-N or placing the cursor in this box with a mouse and clicking the left mouse button will activate this box. The desired file name may be entered or selected from the file list box at this stage. Clicking on OK will select the highlighted file name. Placing the cursor on the down arrow at the bottom of the scroll bar to the right of the file list box and clicking the left button will scroll the list one entry at a time. Placing the cursor at the bottom of the scroll bar, just above the down arrow and clicking the left button will scroll through the box, one page at a time. Users may scroll up through this list in the same manner using the up arrow at the top of the scroll bar. Placing the cursor on a file name and double clicking the left button will select that file for processing.

 Different source and destination drives may be selected. Clicking on the up or down arrows to the right of the drive box will search through the available drives. Positioning the cursor over the desired drive and then clicking the left mouse button once will select the drive.

 Once the source or destination drive is selected, a list of subdirectories appears in the box above the drive box. The cursor may be positioned in this box by clicking the left mouse button. Users may scroll through the list in the same fashion as the file list box. Clicking on OK when the appropriate subdirectory is highlighted will select the subdirectory and generate a list of all files located there with a .TPL extension.

 When the desired file has been selected, click on Ok and the file will be selected for processing. **ELISA** *for Windows* will then continue to the next phase of program execution.

 ELISA *for Windows* will extract the disk drive, subdirectory, and root file name from whatever file is entered in response to these prompts. Thus, if a user enters *file*.DAT located in C:\ELISA\DATA in the data abstraction module, **ELISA** *for Windows* will internally record *file* and the disk drive-subdirectory C:\ELISA\DATA. If the user then immediately enters the parameter estimation module, the file selection dialog window will ask for the name of the standards data file to be processed. **ELISA** *for Windows* will then 'guess' that the proper file and subdirectory location is the one just created in the data abstraction module and insert *file*.STD in the File Name box. The user need only click on OK to select this file. This scheme is used wherever possible throughout the program and greatly facilitates data analysis by eliminating repetitive selection of file names.

Archiving Files

 Outdated files should be archived to floppy diskettes to avoid the necessity of scrolling through overly long lists in file selection dialog windows. When a particular file is archived, **all** files with the same root name should be archived as well. This will keep the linked files together in one group. That is, if it is desired to archive a series of

.DAT files, the associated .STD, .UNK, .QC, .LBL, and .CFT files should be archived as well to keep the file groups intact.

Program Execution

ELISA *for Windows* is initiated by double-clicking the ELISA Windows icon. The opening menu, as shown in Figure 4, will appear. The version number and date will likely be different from that in the figure.

The opening menu consists of a list of options corresponding to program modules. The cursor will appear under the dot (°) in the list of options. The up/down cursor control keys may be used to move through the different options, displaying a short description for each module in the box to the right of the option list. Pressing the ENTER key (or clicking on OK) will select the desired option. Alternatively, clicking the left mouse button once on an option will move the dot to that option and display the descriptive text in the box to the right. Double clicking the left mouse button will select the desired option and execute the associated program module. The remainder of this documentation will deal with each of these modules in detail.
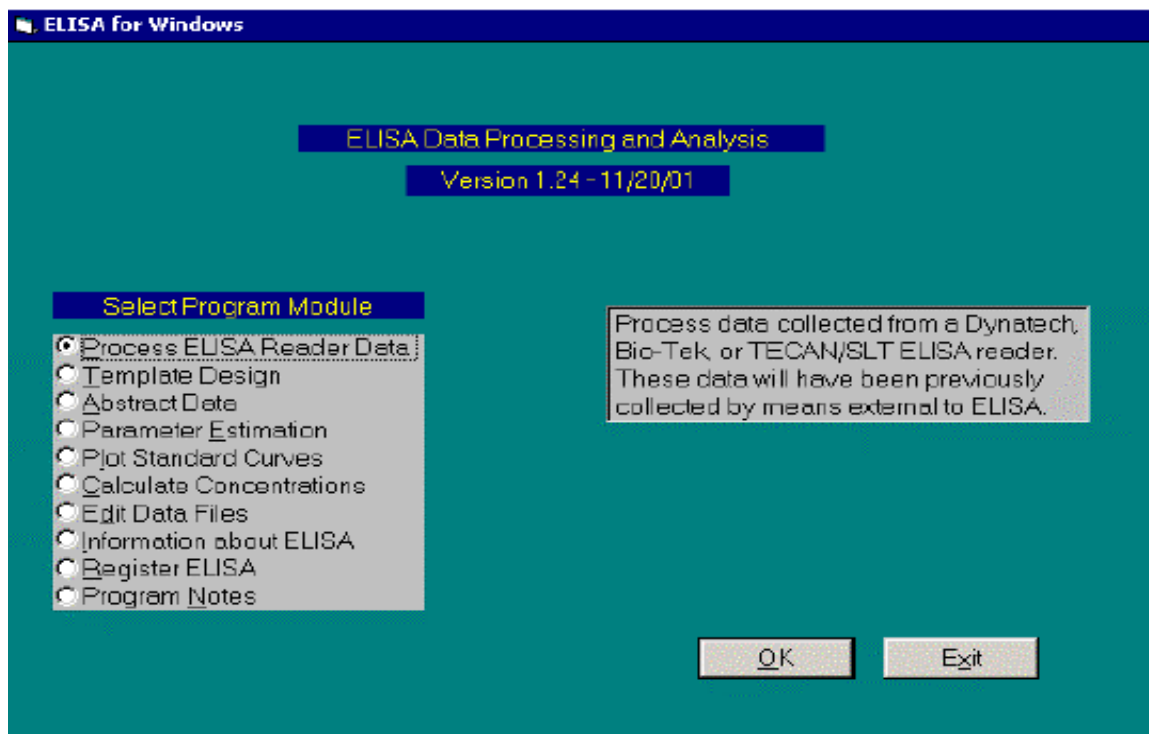
Figure 4. **ELISA** *for Windows* opening menu screen.

MODULE 1

PROCESS ELISA READER DATA

This first module processes data collected from a variety of different ELISA readers and writes these data to *file*.DAT where *file* is specified by the user in response to a file selection dialog window. It is understood that these data have already been captured using some commercial, public domain, or privately created communications package. **ELISA** *for Windows* will **not** collect raw data from these instruments. This module currently processes data collected from a selection of Dynatech and Bio-Tek readers. As our collaborations with other laboratories expand, **ELISA** *for Windows* will be amended to accommodate additional readers.

This module is entered from the main menu (Figure 4). **ELISA** *for Windows* responds with the dialog window shown in Figure 5. At present, the user may select between various Dynatech and Bio-Tek options. The Dynatech translation protocols were created to filter data captured directly from Dynatech readers. The Bio-Tek filters were created to accommodate data captured by programs used by collaborators with the FDA and the University of Rochester. Because these programs slightly alter the data structure, owners of Bio-Tek readers may experience some difficulties when accessing these options. Please contact us if this is happens. New filters may be added to the option list as time and resources permit.
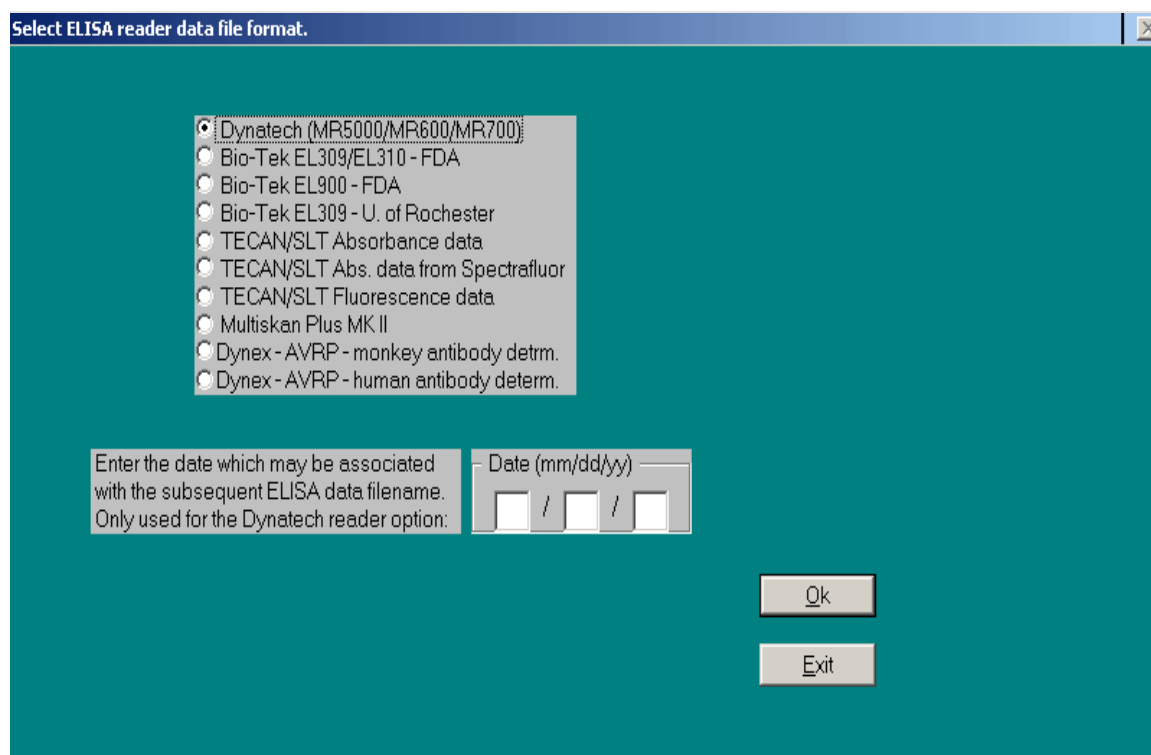
Figure 5. Dialog window for Module 1 - Process ELISA Reader Data.

Dynatech ELISA readers are used within the CRDB laboratories. Figure 6 outlines the format produced by the MR5000. The values in the header records may differ depending on the application and instrument settings used for a particular assay. When capturing the data some communications packages allow the user to enter any number of lines of text to the file. The first few lines of the file outlined in Figure 6 are such comment lines. The MR5000 ELISA reader's actual raw data begin with the MODE REF SAM line. The comment statements are optional and are there to give the user a mechanism for annotating the data. **ELISA** *for Windows* will abstract these comment lines and write them to the .DAT file.

Figure 7 diagrams the data format as collected from the Dynatech MR700. The software that controls this instrument allows the user to enter one line of comments which is the third line of the header records. **ELISA** *for Windows* will abstract only this one line and place it in the processed .DAT file. Any additional comment lines entered by the user during data capture will be ignored.

It is possible for **ELISA** *for Windows* to correctly abstract data from other models of Dynatech readers. If the data conform to one of the two formats outlined in Figure 6 or Figure 7, the program should abstract the data without difficulty. As an example, the older Dynatech MR600 transmits data in the same format as the MR5000 so readings from this older instrument may also be processed by **ELISA** *for Windows*.

The file selection dialog window appears after selecting the data conversion option. Select the file for processing as described in the file selection section in the Introduction of this manual.

We have adopted a particular file naming convention in our laboratories which must be adhered to if the Dynatech option is selected. Raw data files as captured from the ELISA readers are given two letter or digit names without an extension. These files may contain one or more plates of observations. All files two characters in length and without an extension will appear in the file list box if the Dynatech option is selected.

The second through fourth options, dealing with the Bio-Tek readers, assumes the user has completely specified the filename with a .EIA extension. These files may also contain one or more plates. All files with a .EIA extension will appear in the file list box upon selecting one of these two options.

```
comment line 1
comment line 2
.
.
.
 MODE REF SAM
 DUAL  5  1
THRSHLD =    0.00
CAL =        1.00
DATE:_____
OPER:_____
 1 A          1.234
 1 B          1.234
 1 C          1.234
.
.
.
12 H          1.234

Repeat as many plates as desired.
```

Figure 6.   Raw data format - Dynatech MR5000 ELISA reader.

```
Date(/m/d/y):   5/25/91
Plate number:  1
Comment:  This is a comment line
Time:  3:36 PM
Template name:  alk phos
Procedure name:  alk phos
Instrument:  MR700
Mode:  Dual
Test Filter:  1
Reference filter: 1
DATA:
 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234
 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234
 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234
 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234
 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234
 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234
 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234
 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234 1.234
Template:
      1    2    3    4    5    6    7    8    9    10   11   12
A     T1   T9   T17  T25  T33  T41  T49  T57  T65  T73  T81  T89
B     .    .    .    .    .    .    .    .    .    .    .    .
C     .    .    .    .    .    .    .    .    .    .    .    .
D
E
F
G
H     T8   T16  T24  T32  T40  T48  T56  T64  T72  T80  T88  T96
Labels:
T 1:
T 2:
.
.
.
T95:
T96:

Repeat as many plates as desired.
```

Figure 7. Raw data format - Dynatech MR700 ELISA reader.

If the Dynatech option is chosen, suppose, as an example, file BP with two plates is selected. The date is entered next in the format specified (month/day/year). The program will append the date to the two-character raw data file name to create up to an eight character file name with a .DAT extension. Thus, if BP was entered as the two-letter raw data file name, and 11/08/96 was entered as the date of data collection, **ELISA** *for Windows* would create the file name BP110896.DAT as the destination for the processed data.

For the two Bio-Tek readers, the file name is assumed to be fully specified by the user during data capture. Therefore, the date information is not used.

Since a raw data file may contain several plates, **ELISA** *for Windows* will echo the plate number within the raw data file as it is processed:

```
Processing plate number    1
Processing plate number    2
.
.    Continues for all plates
.
```

After all plates have been processed, a message detailing the file name and number of plates contained within the file is displayed:

Data file conversion complete.

Raw data file C:\ELISAWIN\BP processed to yield:
C:\ELISAWIN\BP110896.DAT
File contains 2 plates.

OK

Clicking on OK will return the user to the main menu (Figure 4).

MODULE 2

TEMPLATE DESIGN

This option abstracts three types of data from a 96-well ELISA plate: standards, unknowns, and quality control samples. This option gives the user a wide degree of latitude in creating template formats for this data abstraction. The main restriction involved with this operation is that samples must be arranged in rectangular arrays and like samples (replicates) must be contiguous.

Templates created with earlier versions of **ELISA** may still be accessed but it is strongly recommended that these files be translated to the new format. The simple procedure for doing this is described towards the end of this section. Templates created in this version of **ELISA** *for Windows* will not work in earlier versions of the program.

The template file selection dialog window appears after selecting this module for processing from the main menu (Figure 4). All files with a .TPL extension will appear in the file list box. Select the file for processing as described in the file selection section in the Introduction of this manual. This template will be loaded for viewing and editing.

To create a new template, move the cursor to the File Name box in the template file selection dialog window. Erase all characters in this box with the DELETE key and enter the word 'NEW' (without the quotes) and then click on OK. **ELISA** *for Windows* will respond with the default dialog window shown in Figure 8.

A schematic diagram for a blank plate is displayed along with several boxes to select options and input data. Through the use of these options a template may be designed from scratch or an existing template edited to produce a new template.

The 'Project ID' field allows users to assign a code to the template and may be used to group unknown samples under one study or project identification heading. This field is optional.

In the 'Block Info' box, the user may select one of three types of blocks, standard (Std), unknown (Unk), or quality control (QC). Additionally, the user may edit an existing block or delete a block. The Block ID is another optional mechanism used to assign a unique code to a particular block. As an example, this may be used to code unknowns within a particular project. All subsequent output dealing with the unknown specimens will be annotated with these unique identification codes.

To define a block, position the cursor within the brackets of the upper left cell and click with the left mouse button. An 'X' will appear within the brackets. Repeat this procedure for the lower right cell. Two 'X's will now indicate the opposing corners of the block. Select the type of block just outlined by clicking on 'Std', 'Unk', or 'QC' in the Block Info box. If desired, enter a Block ID. In the 'Dilution Information' box enter the starting dilution (Start) and the magnitude of change between the dilutions in the series (Factor). **ELISA** *for Windows* deals with reciprocal dilutions.

Thus, if a series begins with a 1:600 dilution, 600 would be entered in the Start field. If the series follows a twofold dilution then '2' would be entered in the Factor field. If the series proceeds in an ascending fashion (i.e., 1:600, 1:1200, 1:2400, etc), then select the 'low to high' option. If the series progresses in a descending order (i.e., 1:600, 1:300, 1:150, etc.), then select the 'high to low' option. This may seem backwards at first. The way to conceptualize these prompts is to think of the reciprocal dilution numbers. If there are three dilutions, 600 is less than 2400 so low to high means start at 600 and proceed through 2400. High to low is just the opposite: start with 600 and step down to 150.

In the 'Replicate Information' box the number of replicates and their orientation are selected. **ELISA** *for Windows* will check to make sure these responses are consistent with the block defined by the user. For example, a block defined as A1 - H3 contains eight rows and three columns. This allows for a replicate series running three across (horizontally) or eight down (vertically). Any other responses in these fields will be flagged as an error and the program will ask the user to reenter the required information.

Once all the information necessary to define a block is entered, clicking on the 'Record' command button will save the block definition for later recall and/or editing.

Figure 8. Dialog window for Module 2 - Template Design.

The 'Boundary' field in the Block Info box will contain the coordinates for the upper left and lower right corner cells of the newly defined block. **ELISA** *for Windows* allows only one standards block per plate. After a standards block has been defined, the standards option button in the Block Info box will be inactivated. If an existing standards block is deleted, this option button will be reactivated.

Once a block's information is recorded, the cells are filled in to indicate they are no longer free to be included in succeeding blocks. The cells within a standards block are indicated with an 'S'; unknowns with a 'U'; and quality control samples with a 'Q'. If any block is subsequently deleted, the letters in the corresponding cells will be blanked out indicating the availability of the cells for future block definitions.

To delete a block, click on any cell within the block with the left mouse button. An 'X' will appear in the cell. Select the 'Delete' option in the Block Info box and click on the 'Record' command button. The cells in the block will be blanked out indicating the block has been deleted.

To recall a block's defining parameters, click on any cell within the block with the left mouse button. An 'X' will appear in the cell. Click on the 'Redraw Plate' command button and the information previously entered for that block will be inserted into the appropriate entry fields. Once this information is retrieved, any part of it may be altered. Once the information has been edited, again, click on any cell within the block (confirmed with the placement of the 'X' in the cell) and select the 'Edit' option in the Block Info box. Finally click on the 'Record' button to register the change. To reset or blank out the data entry fields, click on the 'Redraw Plate' command button without first marking a cell in an existing block.

This technique may also be used to copy the contents of one block to another block. This is useful if several unknowns with identical defining parameters are being added to a plate. Once one unknown is established, its information may be recalled and used for any subsequent block's definition. Once the information is inserted in the data entry fields, a new block may be defined by clicking on the opposing corner cells in the plate schematic diagram. Clicking on the 'Record' command button will then establish the new block using all the parameters from the block which was previously recalled. When applying this technique care must be taken to change the Block ID or this information will also be copied over to the new block. This is only necessary if each unknown requires a unique block identifier.

Clicking on the 'View Plate' command button will present an expanded view of the plate with each well defined with its dilution and block designation. Unknowns and quality control samples are numbered in the order in which they are defined. A sample display is shown in Figure 9. If the window is not large enough to contain the entire plate diagram, the scroll bars may be used to pan horizontally and vertically through the display. The block identifiers are listed under the plate diagram and may be viewed using the vertical scroll bar.

It is possible to create a block with a single point dilution. In this case, the user would block off the desired cells and enter the single dilution value in the 'Start' data

entry field. Enter 1 in the 'Factor' data entry field. Finally, enter the necessary data in the Replicate Information box. If there are no replicates (a single point determination in a single well) then enter 1 for the number of replicates and ignore the orientation.

There is no limit to the number of blocks which may be assigned to any one plate with the proviso that there be only one block of standards.

When all blocks of information have been defined, clicking on the 'Save' command button will lead to the template file selection dialog box. The user will be prompted for a filename for the newly created template. A new file name may be specified or the old template file may be over-written and replaced. Retain the .TPL extension to insure the file name will appear in subsequent file name lists when this module is entered in the future. The program will return to the main menu (Figure 4) and the name of the template will appear in the descriptive box to the right of the option group on the opening menu screen when the template design option is selected.

This completes the template creation description. Once the template module is entered from the main menu, all template files will be listed in the file list box in the template file selection dialog window and the user may select the desired template for data processing. Once the selection is made, the formatted template will appear on screen in the context of the dialog window shown in Figure 8.

The 'Select' command button will choose the given template for subsequent

ELISA Plate Configuration - file -> E~-1\BDP1\MYFILES\ELISAWIN\FINAL.TPL											
	1	2	3	4	5	6	7	8	9	10	11	12
A	600	600	600	300	600	1200	2400	4800	9600	19200	38400	1200
	S	S	S	U1	U1	U1	U1	U1	U1	U1	U1	QC1
B	1200	1200	1200	300	600	1200	2400	4800	9600	19200	38400	1200
	S	S	S	U1	U1	U1	U1	U1	U1	U1	U1	QC1
C	2400	2400	2400	300	600	1200	2400	4800	9600	19200	38400	1600
	S	S	S	U2	U2	U2	U2	U2	U2	U2	U2	QC2
D	4800	4800	4800	300	600	1200	2400	4800	9600	19200	38400	1800
	S	S	S	U2	U2	U2	U2	U2	U2	U2	U2	QC2
E	9600	9600	9600	300	600	1200	2400	4800	9600	19200	38400	19200
	S	S	S	U3	U3	U3	U3	U3	U3	U3	U3	QC3
F	19200	19200	19200	300	600	1200	2400	4800	9600	19200	38400	19200
	S	S	S	U3	U3	U3	U3	U3	U3	U3	U3	QC3
G	38400	38400	38400	300	600	1200	2400	4800	9600	19200	38400	0
	S	S	S	U4	U4	U4	U4	U4	U4	U4	U4	
H	76800	76800	76800	300	600	1200	2400	4800	9600	19200	38400	0
	S	S	S	U4	U4	U4	U4	U4	U4	U4	U4	

Project ID: | Test Template | Print   Return

Figure 9. Display of sample template design.

processing in the data abstraction module. 'View Plate' allows the user to examine the chosen template checking the dilution settings within each block of standards, unknowns, and quality control samples. The various options in the Block Info box may be selected to add new blocks to existing templates, delete blocks from templates and change various parameters for any block. In this manner **ELISA** *for Windows* allows users to easily alter existing templates. These editing functions facilitate the creation of new templates that are only slightly different from existing ones without starting from the beginning with a blank template. These editing functions may also be used during the creation of templates to correct entry errors. If a user misspecifies one of the parameters within a block of standards, unknowns or quality control samples, the block may be edited or deleted and recreated. When a template is edited, the user will be given the opportunity to name the new template at the end of the procedure. A template file selection window appears with the original name of the template located in the file name box. Clicking on OK will store the changes in the original template file. The file name may be edited If the user does not wish to overwrite the existing template file.

To translate the format of a template file created with an earlier version of **ELISA** *for Windows* to the current format simply read in the existing template and immediately 'Save' it.

If the user is unsure which template to use, the plate may be queried using the 'View Plate' command button. If it is determined that the template is not the one truly desired, exiting the dialog menu screen with the 'Exit' command button, will return the user to the main menu where the Template module may be reentered and a new template selected.

Never assign a dilution <= 0.0 to any well, for standards, unknowns, or quality control samples. This will result in a fatal error when the concentration calculation module is entered. If dilutions <= 0.0 exist, **ELISA** *for Windows* will end with unpredictable results when calculating these concentrations.

Be careful to click on the 'Record' command button after each operation (defining or editing a block, etc.) to ensure that the action is recorded in the template file. The 'View Plate' option may be used to check the accuracy of each operation

In conclusion, each template is not required to contain blocks of wells representing all three data types. Users are free to create templates with any combination of standards, patient unknowns and quality control samples. The only restriction is that there may be only one block of standards per template.

Refer to Appendix B for a description of the template (.TPL) file format.

MODULE 3

ABSTRACT DATA

This module applies a template protocol to abstract the three different data types, standards, unknowns, and quality control samples, from 96-well ELISA plates stored in plate data files with a .DAT extension. Before executing this option, Module 2 may be used to pick a template, with the 'Select' command button. Refer to the Template Design section for further details.

The present module begins by displaying a data file selection dialog window used to choose a plate data file for processing. All files with a .DAT extension will appear in the file list box. Select the file as described in the file selection section in the Introduction of this manual.

If a template was not selected prior to entering this module, the user will be presented with a template file selection dialog window where a template file may then be chosen.

Once the plate and template data files have been selected, a dialog window appears as is shown in Figure 10. The dialog window displays the name of the plate data

Figure 10. Dialog window for Module 3 - Abstract Data.

file as well as listing the number of plates contained in that file. Also listed is the current template chosen for plate data abstraction. Additionally, the dialog window informs the user of the plate number where processing will begin.

As mentioned in the data file structure section in the Introduction, several plates may be stacked or concatenated in one .DAT file. However, it is not necessary for all of these plates to be analyzed with the same template protocol. Conceivably, the placement of standards, unknowns, and quality control samples may be the same from plate to plate, but the dilution series may differ for some of the plates. Conversely, the different plates within one plate data file may have entirely different configurations. A new template is required each time the dilutions or the plate configuration change. The dialog window in Figure 10 permits the user to abstract the data from each .DAT file using one or more template protocols. If the default option, 'Use template for remainder of file', is selected, each of the three file types will be extracted using the selected template starting with the plate number listed in the dialog window. If the 'Stop after plate number' option is selected, the user will need to enter that plate number in the text box to the right of the option label. If this second option is chosen, the current template protocol will be used to extract the data starting with the plate number the file is currently set to (listed in the dialog window) through plate n where n is the number entered in the text box previously described. Note that the user must first select the 'Stop after plate number' option before actually entering the plate number itself. That is, the program will restrict plate number entry in the text box until the appropriate option has been selected. **ELISA** *for Windows* abstracts the data and then returns to the template file selection dialog window where a new template is chosen. The dialog window in Figure 10 reappears and includes the updated plate pointer: 'Data file currently set to plate number $n+1$'. The user now may choose to apply the new template to the remainder of the file or to stop after another plate number, m, where $m > n$.

To begin, assume the first option is selected where one template will be used for all plates. Once the plate data file has been specified, **ELISA** *for Windows* abstracts the three data types, standards, unknowns, and quality control samples. The abstracted data will scroll up the computer screen. The first column of the screen listing is the dilution in decimal form (e.g., 600 entered in the template module is converted to 0.001666667), the second column is the optical density, and the third column is STD, UNK, or QC for each of the three data types. These three data types will be written to three separate files named using the root file name of the plate data file selected above and the appropriate extension. Thus the standards are written to *file*.STD, the unknowns to *file*.UNK, and the quality control samples to *file*.QC, where *file* is the root file name of the plate data file previously selected. Any comment lines in the .DAT file will be abstracted and written to *file*.LBL. These four files will be written to the same subdirectory as the plate data file. An informational message detailing this will appear as shown in Figure 11.

Clicking on OK will return the user to the main menu (Figure 4).

Figure 11. Concluding message for Module 3 - Abstract Data.

The user selects the second option in the dialog window (Figure 10) to apply more than one template to a group of plates in a .DAT file.

As an example, consider a plate data file with six plates. It is desired that three separate templates be used to process plates 1-3, 4, and 5-6, respectively. The dialog window (Figure 10) initially reports that the file is set to plate 1; select the second option and type 3 in the text box to indicate the current template will process plates 1 through 3. When these plates are processed, the template file selection dialog window will reappear and a new template may be chosen. The dialog window (Figure 10) will then appear stating that the plate data file is now set to plate 4. Select the second option again and type 4 in the text box. After plate 4 is processed, another template is selected and the dialog window reappears. Now the window indicates the plate data file is set to plate 5. choose the first option to process the rest of the plate data file (plates 5 and 6), or select the second option and type 6 in the text window to indicate processing should stop after plate 6. The module will conclude either way with the informational message shown in Figure 11.

If the template employed for abstracting the plate data does not contain one or more of these three data types or if there are no comment lines in the .DAT file, the associated files will still be created. They will, however, be empty. Also, if this module is exited using one of the earlier prompts, these files will also be created and, in this case, each of them will be incomplete. If an empty file is created, do not delete it because, in some cases, its presence may be required by succeeding modules in the data analysis modules of **ELISA** *for Windows*.

MODULE 4

PARAMETER ESTIMATION

This module uses a four parameter logistic-log function to describe standards data and form calibration curves. Standard curves may be formed using two fitting techniques, iteratively unweighted least squares and robust iteratively reweighted least squares. Users may also elect one of two estimation algorithms, Taylor series-linearization or Marquardt's Compromise, to estimate the four parameters. It is beyond the scope of this document to describe these two techniques in detail. Briefly, the unweighted least squares method estimates the parameters without taking into account the possible differences in the variability of optical density measurements as one moves from dilution to dilution. Recognizing that this fit may be unduly influenced by aberrant observations (outliers), the robust fitting technique weights each observation individually and inversely proportionally to the residual difference between the observed and predicted values for each point. In this way, outliers are assigned low weights and exert little influence in the overall fit of the curve to the data.

Interested readers may refer to reference 7 (listed at the end of this section) for a further discussion of the theoretical details. Note that in their article, Tiede and Pagano refer to the four parameter function as a 'modified hyperbola.' In fact, it is algebraically equivalent to the logistic-log model. References 4 - 6 provide additional articles of interest. Our philosophy governing the use of these models in assay methodology is described in reference 1. For a discussion of the Taylor series-linearization and Marquardt's Compromise estimation algorithm, readers are referred to Chapter 10, an introduction to nonlinear estimation, in reference 2. Reference 3 describes the Marquardt's Compromise technique in detail.

Users should be aware of the following broad generalizations when deciding which fitting algorithm to use. The Marquardt's Compromise algorithm may, at times, expend more calculations, take more CPU time to arrive at a final set of estimates, and involve more iterations than does the Taylor series-linearization technique. The iterative steps tend to be smaller, especially as the converging process nears an end. However, this algorithm is more elaborate and is not as sensitive to poor starting values as the Taylor series technique. Marquardt's Compromise is usually used when the Taylor series-linearization process fails to converge to a solution.

Since the model describing the standards data is the four parameter logistic-log function, a minimum of five data points is required for parameter estimation. Note that this does not mean there need be a minimum of five separate dilutions, just five data points spread out over some number of dilutions. Of course, if the data do not adhere to some semblance of a sigmoidal, S-shaped curve, this procedure will fail. It is the responsibility of the user to select a dilution schedule that will insure that there is enough information for **ELISA** *for Windows* to successfully estimate the four parameters.

A third fitting technique, the cubic spline, is also implemented in **ELISA** *for Windows*. This method is accessed directly in the 'Plot Standard Curves' (Module 5) and 'Calculate Concentrations' (Module 6) segments of the program and is discussed in further detail in those sections of this documentation. The spline fit is a useful technique in those cases where the parametric logistic-log function will not converge to a solution. This is often the case when the standards do not fully describe a sigmoidal-shaped curve. It is inherently difficult to arrive at reliable parameter estimates for the logistic-log function without a clear indication of the minimum and maximum optical densities associated with the assay. That is, if it is difficult to discern both lower and upper asymptotes for the sigmoidal-shaped curve (areas where the curve flattens out), then the logistic-log function fitting techniques may not converge to a solution. In this case, the spline fit may be used to interpolate antibody concentrations for unknown patient samples in Module 6.

Standards File Selection

This module is entered from the main menu (Figure 4) and begins by displaying a standards data file selection dialog window. If the user enters this module directly after abstracting the data from a .DAT file (Module 3), **ELISA** *for Windows* will insert a file name in the file name box which corresponds to the .DAT file just processed. This is offered as a convenience to the user in an attempt to eliminate repetitive entry of file names. This file is selected by clicking on OK. The user may also select a different file from the file list box as described in the file selection section in the Introduction of this manual.

Once the standards data file has been selected, **ELISA** *for Windows* responds with the dialog window shown in Figure 12.

The default selections in this dialog window include using the Marquardt's compromise estimation algorithm with the robust fitting technique. All output will be directed towards the printer. The entire standards data file will be processed without interruption with each step using up to 100 iterations. The starting values for the iterative estimation process will be calculated by the program.

Users may alter these default settings to best handle their data sets. The estimation techniques are options and, as such, only one may be selected. The destination of the results are also options and users may select either the printer, computer screen, or file. If the screen is selected then the results of each plate are displayed on screen. Users then have the option of exiting to the next plate or printing the contents of the screen. If 'file' is selected, the user will be prompted for the name of a file to save the results. **ELISA** *for Windows* will suggest *file*.FIT, where *file* is the root file name of the standards data file. If the user selects the printer or file, the standards data will be processed without interruption with all output directed towards the printer or the designated file. The type of least squares fit are marked with check

boxes. As such, robust, unweighted least squares, or both may be selected. If the user inadvertently neglects to select a type of fit, the robust fit is used as the default.

Coefficient Update

Users have the opportunity to enter the proportion for coefficient update. Note that this parameter is only valid when the Taylor series estimation algorithm is selected. If the Marquardt's Compromise algorithm is chosen then this option is suppressed, as the Marquardt technique calculates the update factor automatically. This number must be greater than 0.0 and less than or equal to 1.0. We have found that the value 0.4 works well for our applications and is the default value which appears in the text box in the dialog window when the Taylor series estimation technique is selected. If desired, the user may alter this value. The program may occasionally fail to converge to a solution with some data sets. Outliers will severely affect the outcome of the algorithm used to calculate initial starting values for the iterative fitting procedure. In this case, coefficients from a previously successful run with similar data may be substituted for the calculated starting values and the iterative

Figure 12. Dialog window for Module 4 - Parameter Estimation.

process retried. Alternatively, the proportion for the coefficient vector update can be modified. This is seen more clearly if one examines the weighted least squares equation:

$$\mathbf{O}_{j+1} = \mathbf{O}_j + (\mathbf{A}_j^T\mathbf{W}_j\mathbf{A}_j)^{-1}\mathbf{A}_j^T\mathbf{W}_j\mathbf{R}_j$$

and re-expresses it as:

$$\mathbf{O}_{j+1} = \mathbf{O}_j + \mathbf{B}_j$$

where $\mathbf{B}_j = (\mathbf{A}_j^T\mathbf{W}_j\mathbf{A}_j)^{-1}\mathbf{A}_j^T\mathbf{W}_j\mathbf{R}_j$

The most recent estimate for the parameter vector $\mathbf{O} = \mathbf{O}_{j+1}$ is equal to the last estimate, $\mathbf{O}_j$, plus an amount equal to $\mathbf{B}_j$. If $\mathbf{B}_j$ is very large the solution may circle around some value without actually converging, or it may rapidly expand and diverge to the point of causing a numeric overflow in the program. To avoid this, the magnitude of $\mathbf{B}_j$ may be decreased by altering the proportion for the coefficient vector update. If the user enters 1.0 then the result is the standard equations listed above. If the solution does not converge or if it diverges to numeric overflow, a smaller proportion, e.g. 0.4, may be used. This would modify the second equation, above, to:

$$\mathbf{O}_{j+1} = \mathbf{O}_j + (0.4)\mathbf{B}_j$$

The proportion may be altered at will to compute smaller and smaller increments of change for the $\mathbf{O}_j$ vector. **ELISA** *for Windows* suggests a starting value of 0.4 and uses this as its default entry. The user is free to experiment with this value. Numbers close to 1.0 will result in larger steps between iterations and may lead to faster convergence (it may also lead to faster *di*vergence as well). Numbers close to 0.0 will lead to smaller steps between iterations and lead to a slower convergence. This may be necessary for difficult data sets.

Number of Iterations

If the coefficient for vector update is small (less that 0.4), then the iterative increments tend to be smaller, requiring more steps to reach a stable set of coefficients. The default number of iterations is 100 and this may be increased to accommodate these situations. This is particularly true for those instances where the standards data do not fully describe a sigmoidal-shaped curve causing the iterative steps to proceed slowly to a final solution.

Note that the default setting for the number of iterations is set to 100. If the program is left unattended and loops around a solution, it will print out the results of

the 100th iteration as though this were the final answer. Take care to examine the output. If the number of iterations is listed as 100 or 101, or, if the number is listed as that which was entered by the user, then the solution may be suspect. If this occurs, the user should examine the standards data as well as the fitted calibration curve using Module 5 - 'Plotting Standard Curves'.

Pause after Iteration

The user may elect to view the results of each iteration by checking the 'Pause after each iteration' box. Coefficients with the resulting sum of squares of residuals and correlation coefficient are displayed on screen after each step of the fitting procedure. A general guideline as to whether or not a revised set of coefficients is performing better than the previous set is the sum of squares of the residuals which should decrease with each successive iteration. If the number steadily increases, this is an indication that the solution is starting to diverge. The program may still converge at a later stage or it may continue to diverge until a numeric overflow occurs. If it decreases then increases and starts to decrease again in a cyclic fashion, this is an indication that the program is circling around a solution without actually being able to achieve convergence.

It is important to note that there are two sums of squares of residuals returned for the approximate least squares fit and the robust fit. The regular sum of squares is simply the sum of the squared residuals. When fitting a simple linear model to a data set, this is the quantity that is minimized when solutions for the coefficients are found. In the case where a nonlinear function such as the four parameter logistic-log model is being fit using the Taylor series expansion method, an approximation to this quantity (the approximate sum of squares) is actually minimized when the coefficients are solved. As the solution converges, notice that these two quantities approach each other in magnitude. They should be fairly close to each other if the process concludes successfully. Interested users are referred to Chapter 10 of reference 2 - pages 462-465 for further details. In particular, equation 10.1.5 details the regular sum of squares of the residuals and equation 10.2.7 the approximation to this quantity resulting from the Taylor series expansion. Note the four coefficients change with smaller and smaller increments as they also converge to their final estimated values.

At the end of each iteration, **ELISA** *for Windows* will pause and display information as shown in Figure 13. If this particular iteration is satisfactory, and the user wishes to end the process, selecting 'No' will conclude the estimation process for the current plate, record the coefficients as detailed on screen, and proceed to the next plate. Selecting 'Yes' will initiate another iteration step. This process cycles until **ELISA** *for Windows* iterates to its own solution or the user stops the process by selecting 'No'.

Estimation Starting Values

Good starting values are essential for the success of any nonlinear iterative fitting process. We have incorporated an algorithm which computes starting values for the iterative process used by **ELISA** *for Windows* to estimate the four parameters of the logistic-log function (see reference 4). Occasionally, these values will not lead to a solution, that is, the process will diverge away from a solution or circle around a solution without actually settling in on one set of values which may be used for parameter estimates. This is especially true if there are outliers in the data or if there are a small number of data points. **ELISA** *for Windows* gives the user the option of overriding the automatically calculated starting values so that a set of possibly more accurate values from a previously run standards file may be used in their place.

If the user elects to input starting values, the box for this option must be checked, otherwise, coefficient data entry will be suppressed. One or more starting values for coefficient estimation may then be entered. **ELISA** *for Windows* will insert the automatically calculated starting values in those instances where the user did not manually enter them. As an example, one may manually enter starting values for the first two coefficients (A and B) and leave the remaining coefficients (C and D) blank. The program will access the user inputted values for A and B and internally calculate values for C and D.

```
COMPUTING ROBUST AND LEAST SQUARES LOGISTIC CURVE COEFFICIENTS
            (Taylor Series-Linearization algorithm)
         Processing file: C:\ELISA\BD010292.STD  Plate: 1
           Results stored to file: C:\ELISA\BD010292.FIT
                        Robust Fit
              Coefficient vector update: .4
 ================================================================

  +-|            Continue coefficient estimation?            |-+
  |                                                            |
  |  Yes -> continue with another estimation iteration.        |
  |  No  -> exit and save current estimates of coefficients.   |
  |                                                            |
  |              +=======+  +--------+                         |
  |              || Yes ||  |  No    |                         |
  |              +=======+  +--------+                         |
  +------------------------------------------------------------+


   Iteration        A          B          C          D
      2         11.22451   -11.22593    0.00204    1.01412
   Regular and Adjusted sum of squares of the residuals
            4.75814D-03      4.27754D-03
   Approximate multiple R^2 =   0.9991
```

Figure 13. Module 4 - 'Pause after each iteration' screen.

At the conclusion of this module **ELISA** *for Windows* informs the user of the creation of the coefficient file, as shown in Figure 14.

Clicking on OK will return the user to the main menu (Figure 4).

Solution Nonconvergence

ELISA *for Windows*, will complete process a standards data file even when one or more plates within the file contain data where coefficients may not be estimated. In those instances the output will be annotated with the message 'Solution did not converge' on the right side of the listing (described below). In this fashion the program will not end midstream while analyzing a standards file containing many plates. **ELISA** *for Windows* will keep track of those plates where convergence was not achieved. In succeeding modules, these situations are addressed by offering the spline fit as the only option for plotting standard curves or calculating antibody concentrations.

Analysis Strategies

Through the various options just outlined, an analysis strategy could proceed as follows: Select the Taylor series estimation technique with 0.4 for the proportion for the coefficient vector update. Choose the desired type of fit. The program will proceed through the selected type of fit for each set of standards in the .STD file.

There are a variety of strategies to employ if the program does not converge to a solution. If the starting values generated from **ELISA** *for Windows*'s internal algorithm are distant enough from the true solution, the fitting procedure may not converge to a final set of values. Users should try the Marquardt's Compromise algorithm if the Taylor series technique fails.

When the program fails to converge, the investigator should first try to select coefficients from a previously successful run with similar data and substitute them for
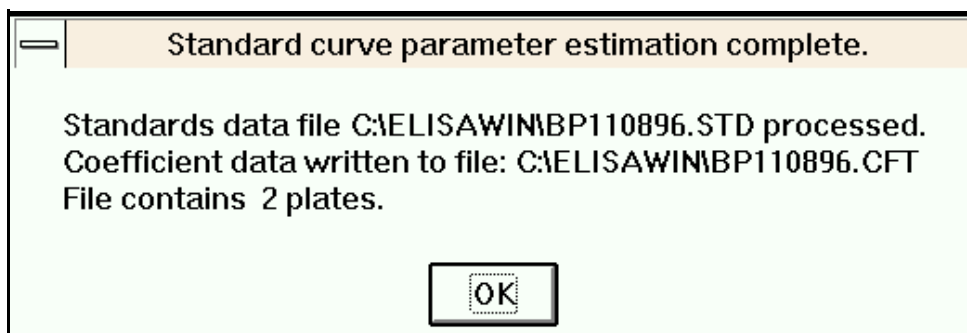
Figure 14. Concluding message for Module 4 - Parameter Estimation.

the calculated starting values through the manual entry of starting values detailed previously. If such values are unavailable, the investigator should rerun the program choosing a smaller proportion for the coefficient vector update - say 0.3 or 0.2. As the program proceeds, the user should inspect the coefficients and the sum of squares of the residuals. The coefficients should stabilize and the sum of squares decrease.

If the solution does not converge but circles around a solution without actually converging, again, examine the sum of squares of the residuals that is returned after each iteration. The coefficients that return the smallest sum of squares can be used as starting values for another attempt at finding a solution. When re-executing the program, manually enter these coefficients and select a smaller proportion for the coefficient vector update than was used previously, and monitor the iterative process.

If the solution continues to circle around a set of values without actually converging, select those values which return the smallest sum of squares of the residuals and test them examining the goodness of fit statistics. If the error rates are acceptable, these coefficients may be used to form the standard curve. These results may also be examined graphically using Module 5, which produces a logistic-log plot.

Output Interpretation

Figure 15 contains an abbreviated listing of output generated from this module (Refer to page 56 for a sample of a complete listing). A complete listing for a sample data file is included in Appendix A. The logistic model used in **ELISA** *for Windows* is parameterized as:

$$y_i = a + \frac{b}{1 + c(x_i^d)}$$

Where **a** = the upper asymptote of the sigmoidal curve, **a** + **b** = the lower asymptote, **c** is related to the dilution at the midpoint of the assay, and **d** is a curvature parameter, related to the slope of the curve; y_i = the *I* th optical density and x_i = the *I* th relative dilution (individual sample dilution / maximum dilution in the series) multiplied by 100.0.

The output will list the file name of the standards file and the plate position within the file along with the type of fit, least squares or robust. Recall that a standards (.STD) file may include several plates of standards. Next the output will list the number of iterations required to reach a solution. Again, if this number is at the maximum set by the user, the results are suspect. **ELISA** *for Windows* simply stopped the iterative process after the maximum possible iterations and chose the last set of coefficients as the final set. The starting values for the coefficients used in the iterative process are listed next.

A table of values follows for each of the standards. The first column is the sample dilution. The second column is the relative dilution calculated as:

((sample dilution/maximum dilution) × 100).

The measured or known optical density occupies the third column followed by the predicted optical density from the fitted curve. The residual is calculated as:
the known OD - predicted OD.

The % error equals the absolute value of:

(residual / known OD) × 100.0.

```
Results from 4 parameter logistic-log fit

File = C:\DOCUME~1\BDP1\MYFILES\ELISAWIN\MA112190.STD - Plate: 1
 Project ID = Test Template / Standards ID = Std 1
 Date: 12/20/2001   Time: 15:43:11
:FRASCH ASSAY PB2 1:75 PLATE 1 UNKNOWNS ARE PB2 1:75
Results of approximate least squares fit
The procedure used 7 iterations.

Starting values for the iterative process:
     2.28081    -2.32942     .03409    1.00000

                 Rel.     Known    Pred.
      Dil.       Dil.     O.D.     O.D.      Residual   % Error    Weight

   0.001666667   100.00   1.716    1.7559    -0.040     2.325      1.0000
   0.001666667   100.00   1.757    1.7559     0.001     0.063      1.0000
   0.001666667   100.00   1.769    1.7559     0.013     0.741      1.0000
   0.000833333    50.00   1.438    1.4315     0.007     0.453      1.0000
   0.000833333    50.00   1.462    1.4315     0.031     2.087      1.0000
   0.000833333    50.00   1.458    1.4315     0.027     1.818      1.0000
   0.000416667    25.00   0.967    1.0095    -0.042     4.391      1.0000
   0.000416667    25.00   1.014    1.0095     0.005     0.448      1.0000
   0.000416667    25.00   0.998    1.0095    -0.011     1.148      1.0000
   0.000208333    12.50   0.586    0.6097    -0.024     4.047      1.0000
   0.000208333    12.50   0.606    0.6097    -0.004     0.613      1.0000
   0.000208333    12.50   0.627    0.6097     0.017     2.757      1.0000
```
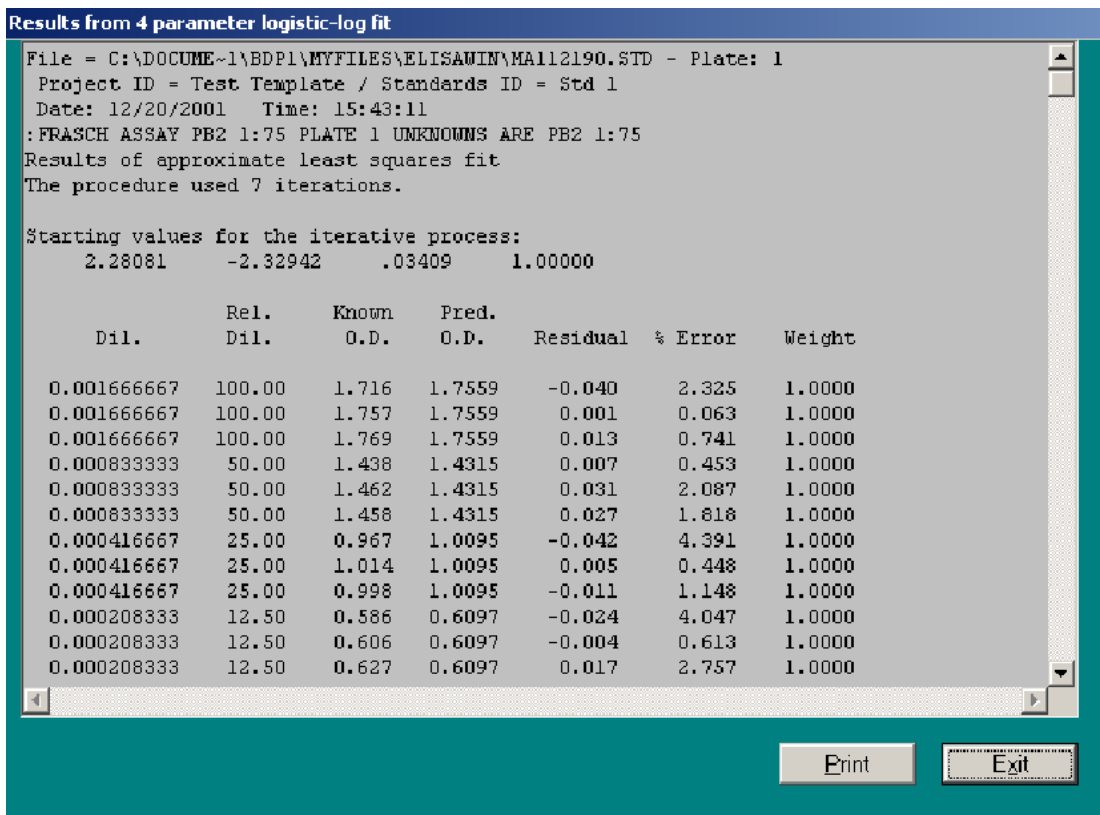
Figure 15. Sample output from Module 4 - Parameter Estimation.

Note that the % error may not precisely equal this quantity when performed on a hand-held calculator. This is due to roundoff error since **ELISA** *for Windows* performs most of these calculations in double precision and retains many more significant digits than are displayed in the output window. The weight column lists the individual weights used for the observation in the final fit of the logistic-log model to the data. For the least squares fit this weight will be set at 1.00 for all observations, indicating that each data point exerted equal influence in the final fit of the model to the data. This essentially is the result of an unweighted fit. For the robust fit, these weights will range from 0.0 to 1.0. The closer the weight is to 0.0, the less influence that point will exert on the fit. The points with weights closer to 1.0 will exert much more influence in the final fit than points with weights close to 0.0. Points with low weights may be considered potential outliers.

The final estimates for the four coefficients follow in order of **a**, **b**, **c**, and **d** (refer to listing in Appendix A). The regular and adjusted sums of squares, as well as the approximate multiple R^2 follow. For the least squares fit, the variances of the four coefficients are reported.

Typically, the window used to display the results will not be large enough to frame the entire listing. There are horizontal and vertical scroll bars on the bottom and right side of the window, respectively. These scroll bars work the same way as the file list box described in the file selections section of the Introduction. Clicking the left mouse key on the down arrow of the vertical scroll bar will advance the listing one line. Clicking just above the arrow key will advance the listing by one screen. The horizontal scroll bar is used in the same manner to shift the listing left and right.

It should be noted that users may edit the contents of the output window. Comments may be added to further annotate the output. Several rudimentary Microsoft Word operations will work in these windows. For example, text may be marked by clicking the left mouse key and dragging the cursor through the target text or pressing the shift key while moving the cursor through the text with the cursor control keys. Once text is marked it may be deleted, moved, etc.

After reviewing the contents of the output window, clicking on 'Exit' will erase the screen and proceed to the next round of estimation with a new plate or additional type of fit. Clicking on 'Print' will send the contents of the output window to the printer. If edits were made in the output window, these changes will be reflected in the printed outputs. They will not be saved once the user clicks on 'Exit'.

References

(1) C. M. Black, B. D. Plikaytis, T. W. Wells, R. M. Ramirez, G. M. Carlone, B. A. Chilmonczyk and C. B. Reimer, Two-site immunoenzymometric assays for serum IgG subclass infant/mother ratios at full term, (1988) J. Immunol. Methods, **106**:71-81.

(2) N. R. Draper and H. Smith, Applied Regression Analysis, second edition, (1981), John Wiley & Sons, Inc., New York.

(3) D. W. Marquardt, An algorithm for least squares estimation of nonlinear parameters, J. Society for Industrial & Applied Mathematics, (1963) **11**:431-441

(4) B. D. Plikaytis, G. M. Carlone, P. Edmonds, and L. W. Mayer, Robust estimation of standard curves for protein molecular weight and linear-duplex DNA base-pair number after gel electrophoresis, (1986) Anal. Biochem., **152**:346-364.

(5) B. D. Plikaytis, P. F. Holder, L. B. Pais, S. E. Maslanka, L. L. Gheesling, and G. M. Carlone, Determination of Parallelism and Nonparallelism in Bioassay Dilution Curves, (1994) J. Clin. Microbiol, **32**:2441-2447.

(6) B. D. Plikaytis, S. H. Turner, L. L. Gheesling, and G. M. Carlone, Comparisons of standard curve-fitting methods to quantitate *Neisseria meningitidis* Group A polysaccharide antibody levels by enzyme-linked immunosorbent assay, (1991) J. Clin. Microbiol., **29**:1439-1446.

(7) J. J. Tiede and M. Pagano, The application of robust calibration to radioimmunoassay, (1979) Biometrics **35**:567-574.

MODULE 5

PLOT STANDARD CURVES

This module forms a fully annotated plot of the points contained in a standards data file along with the fitted standard curve. This module will access the coefficient data file to determine which fitting techniques were chosen during parameter estimation and only offer these for plotting. Thus, if only the robust fit was selected in Module 4, the user would not have the option of selecting unweighted least squares for plotting.

A nonparametric fit, the three point cubic spline, is also offered as an additional option which is always available for selection. The standard curve line described by the spline fit is programmed to pass through the median value of the replicate optical densities at each dilution along the curve. The user may enter this module prior to parameter estimation if it is desired to view the shape of the data in the standards data file before an attempt is made to fit the logistic-log function. In this case, the spline fit will be the only option available. In addition, if there were any plates in a standards data file where the solution did not converge to a stable set of parameter estimates after processing the data with Module 4, then the spline fit, again, will be the only option available for these plates.

These standard curve plots may be directed to the screen and any printer addressed through the Windows interface. The user may direct a single plot or an entire file of standard curve plots to the printer.

This module is entered from the main menu (Figure 4) and begins by displaying a standards data file selection dialog window. If the user enters this module directly after abstracting the data from a .DAT file (Module 3), or estimating the logistic-log parameters (Module 4), **ELISA** *for Windows* will insert a file name in the file name box which corresponds to the .DAT or .STD file just processed. This is offered as a convenience to the user in an attempt to eliminate repetitive entry of file names. This file is selected by clicking on OK. The user may also select a different file from the file list box as described in the file selection section in the Introduction of this manual.

After a standards data file has been chosen, the dialog window in Figure 16 appears. The window lists the name of the file and the number of plates contained in the file. The user should enter the desired plate in the appropriate text box and then exit this field using the Tab key or clicking on the type of fit option box. When the plate number field is exited, **ELISA** *for Windows* will interrogate the coefficient file associated with the standards data file and determine which types of fit should be made available for selection for that particular plate. Fits which were not used during parameter estimation will be suppressed as options here. If a coefficient file does not exist then the spline fit will be the only available option. As stated earlier, when a
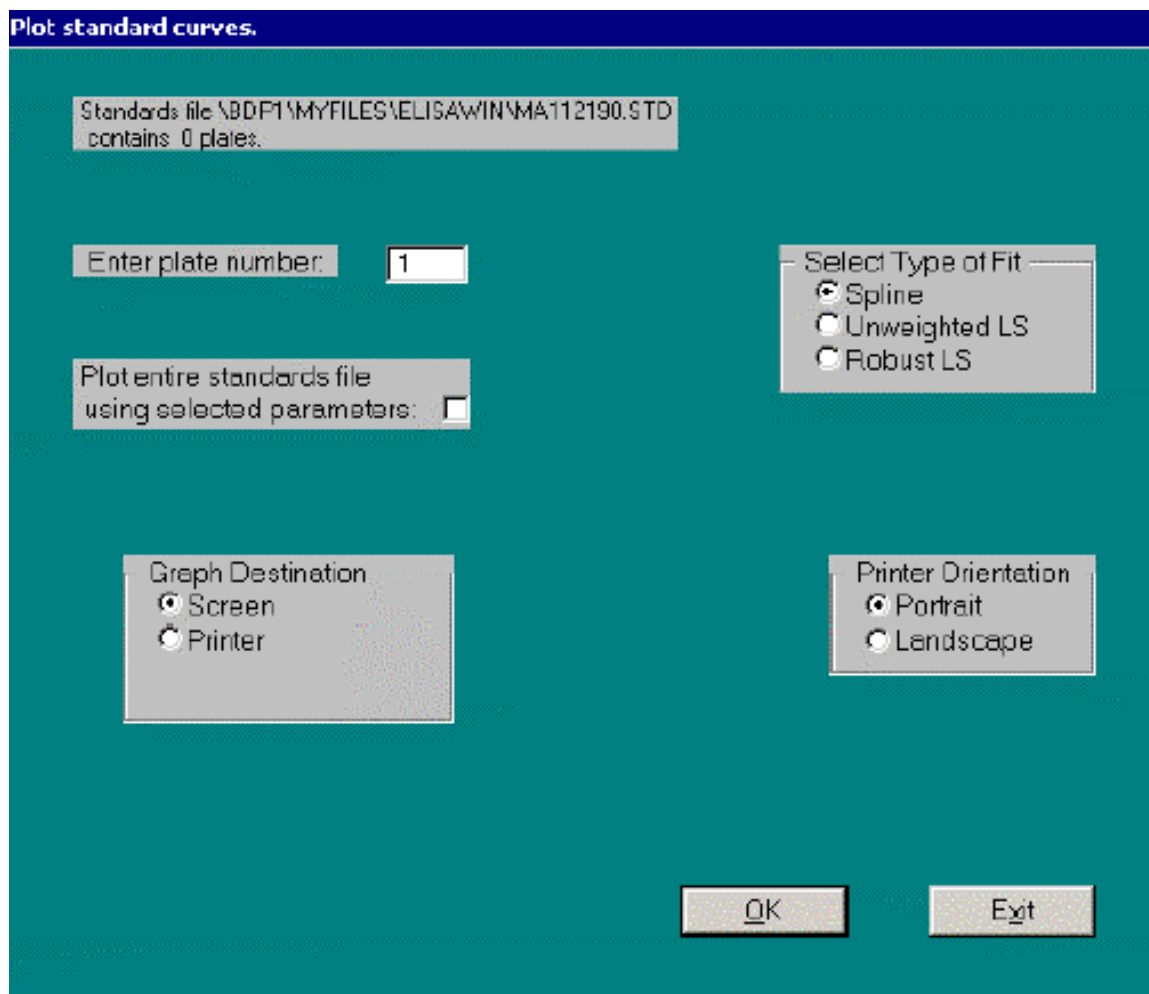
Figure 16. Dialog window for Module 5 - Plot Standard Curves.

coefficient file does exist, the spline fit is the only method allowed for those plates where the solution did not converge during parameter estimation.

If the user selected the printer as the graph destinations, **ELISA** *for Windows* will display the plot onscreen and then automatically send the screen image to the printer without user intervention. If the entire file is selected for processing, the printer is the only allowed destination. The user will be presented with all options for type of fit. If the program was unable to estimate coefficients for the standard curve for one or more of the plates in the file, the spline fit will automatically be chosen for these when they are displayed to the screen and sent to the printer. It is important to note that for this version of **ELISA** *for Windows*, the number of colors for the Windows environment must be set to 256 or less. If the number of colors is set to a higher value (e.g. 65K), the plot will be displayed onscreen correctly but an error will occur if the screen image is directed to the printer. This setting is altered using the screen properties dialog

in Windows 95, the Control Panel in Windows 3.x or whatever software accompanies the graphics display board installed in a particular PC.

If the screen is selected as the destination for the graph, a plot will follow with the type of fit and standards file name appearing at the top of the screen. The printer orientation will not affect the screen image, only the resultant printout. A typical plot will appear as shown in Figure 17. The 95% confidence intervals are included with the unweighted least squares fit. The user may scroll through the other standards series in the file by clicking on the Next and Previous buttons. Each plot may be directed to the printer at this stage. If the user elected to plot the entire file, each one will transmitted to the printer automatically, without user intervention.

The user may choose another plate for standard curve plotting or exit to the main menu (Figure 4).
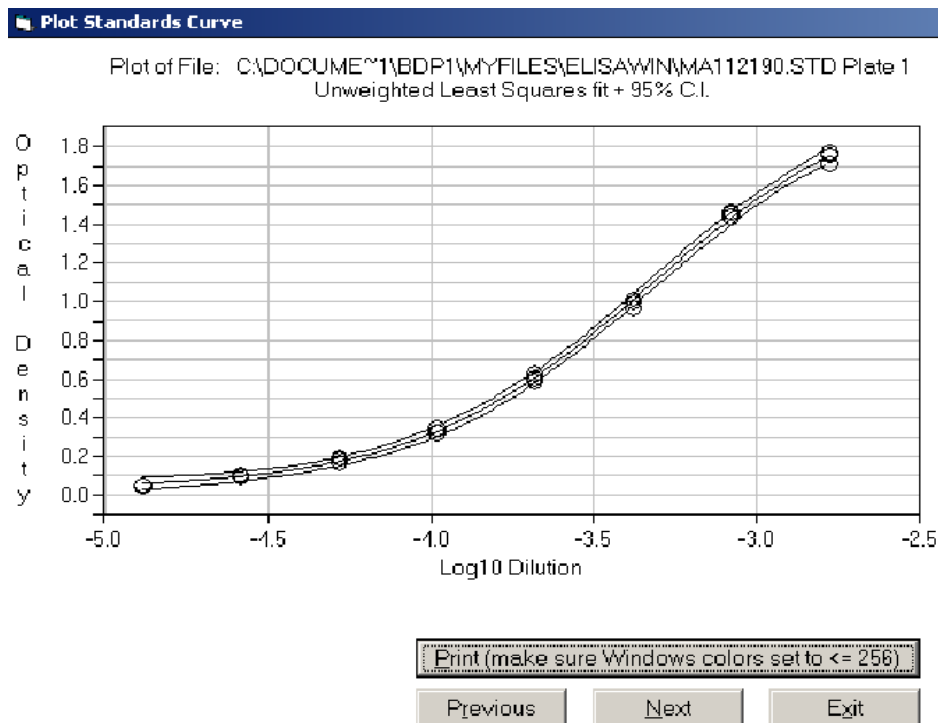
Figure 17. Typical plot of standards data overlaid with fitted standard curve.

MODULE 6

CALCULATE CONCENTRATIONS

Introduction

This module facilitates calculating unknown antibody concentrations from patient unknowns and quality control samples. These concentrations may be arbitrarily assigned units (e.g., antibody units/ml), or mass values (e.g., µg/ml). A diagram used to illustrate the concentration calculations is shown in Figure 18. In this diagram, the absorbance is plotted on the logit-log scale to simplify the example. **ELISA** *for Windows* performs the actual calculations using the results of the more accurate four parameter logistic-log fit.

Figure 18 displays a rather idealized standard curve bracketed by two twofold serially diluted patient unknown curves. For the purposes of this example, assume the antibody concentration of the standard reference serum (the calibration factor) is 135.8
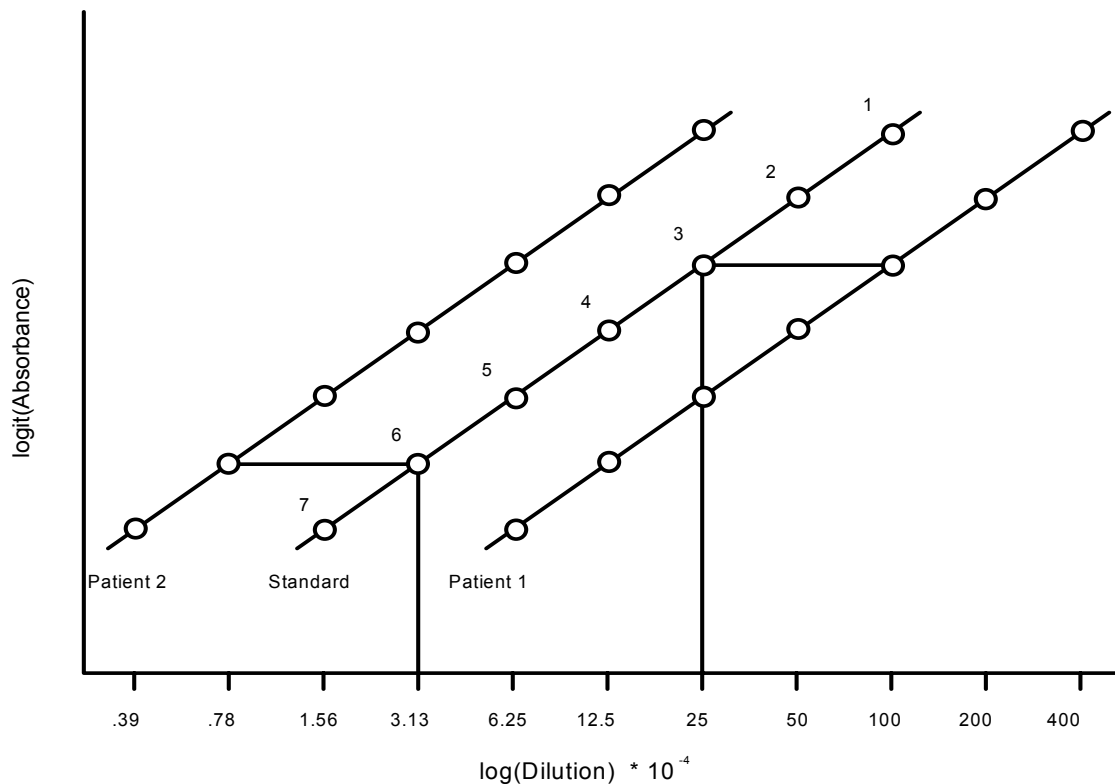
Figure 18. Diagram illustrating antibody concentration calculation for patient unknowns.

micrograms/ml (μg/ml), and that the standard was initially diluted 1/100 with six subsequent twofold serial dilutions. This would lead to standard point 1 containing 1.358 μg/ml, point 2 containing 0.679 μg/ml, etc. The sample from Patient 1 was initially diluted 1/400 and that from Patient 2 was initially diluted at 1/25.

ELISA *for Windows* uses the following formula to calculate antibody concentration:

$$\text{concentration} = \frac{\text{interpolated dilution from standard curve}}{\text{actual dilution of sample}} \times \text{calibration factor}$$

The calculated concentration for Patient 1, using the third point on the patient curve would be:

$$33.95\ \mu g/ml = \frac{0.0025}{0.01} \times 135.8$$

Alternatively, the calculated antibody concentration for patient 2, using the sixth point on the patient curve would be:

$$543.20\ \mu g/ml = \frac{0.0003125}{0.000078125} \times 135.8$$

The optical densities and dilutions corresponding to patient and quality control samples are located in the *file*.UNK (patient unknowns), and *file*.QC (quality control samples), where *file* is the root file name associated with the collection of samples.

The user **must** perform parameter estimation (Module 4) prior to entering this module. This module will access the coefficient data file to determine which fitting techniques were chosen during parameter estimation and only offer these for antibody concentration calculations. Thus, if only the robust fit were selected in Module 4, the user would not have the option of selecting unweighted least squares for calculation purposes. **ELISA** *for Windows*, also offers a nonparametric fit, the three point cubic spline, as an additional option which is always available for selection. If there were any plates in a standards data file where the solution did not converge to a stable set of parameter estimates after processing the data with Module 4, then the spline fit, again, will be the only option available for these plates.

Opening Menu Dialog

This module is entered from the main menu (Figure 4) and begins by displaying a standards data file selection dialog window. If the user enters this module directly after abstracting the data from a .DAT file (Module 3), or estimating the logistic-log parameters (Module 4), **ELISA** *for Windows* will insert a file name in the file name box which corresponds to the .DAT or .STD file just processed. This is offered as a convenience to the user in an attempt to eliminate repetitive entry of file names. This file is selected by clicking on OK. The user may also select a different file from the file list box as described in the file selection section of the Introduction of this manual.

After a standards data file has been chosen, the dialog window in Figure 19 appears.

The window displays the standards data file name along with the number of plates contained in the file. Initially, the window is set to plate 1, unknown 1. The number of unknowns within each plate is also listed. Users may then select the plate and unknown for antibody concentration calculations. When a new plate number is entered in the plate number field, **ELISA** *for Windows* will interrogate the unknowns data file to determine how many unknowns are contained on that plate and display this in the dialog window. This information is updated when the plate number field is exited.

When the user exits the plate number field, **ELISA** *for Windows* also interrogates the coefficient file associated with the standards data file and determines which types of fit should be made available for selection. Fits which were not used during parameter estimation (Module 4) will be suppressed as options in the type of fit option box. As mentioned earlier, the spline fit will be the only choice allowed for those plates where the solution did not converge during parameter estimation.

Users may analyze an entire plate of unknowns or an entire file of plates without returning to the dialog window. In these cases, the parameters set initially (type of fit, calibration factor, etc) will apply for the entire plate or file. By default, when either of these two options are selected, the program will save the results to a report file and not display them on screen.

The construction of the report is designed using the choices in the 'Select computation parameters' box. Unknowns and quality control samples may be estimated alone or together depending on which boxes are checked. If a particular plate does not contain unknowns or quality control samples, these check boxes will be suppressed. If both boxes are checked, the QC samples will follow the unknowns in the output file described later. Next, the calibration factor **must** be entered - **ELISA** *for Windows* will not exit this dialog window without a number specified for this quantity. If a calibration factor is not needed, enter 1 in this box.

The user may elect to save the results of the subsequent calculations to a report file by checking the 'Save report to file (.RPT)' box. This output file will remain in effect and open throughout the procedure, that is, until the user exits the module and

returns to the main menu. **ELISA** *for Windows* will annotate the output with the comment lines contained in the *file*.LBL file. An additional title for the report may be entered in the dialog window.

   The user also has the option of storing a distilled version of the output to a summary file which has an assigned .SUM extension. The summary file is an abridged version of the report file. This file abstracts pertinent information about each plate, unknown, and QC sample, eliminating much of the detail included in the report file. This file contains the overall mean for each unknown, excluding the dilution-specific information. However, dilution means are recorded for quality control samples. The report file is cummulative in nature. Each result is appended to the end of this file. The summary file is updated in that information is revised as users censor optical densities and recalculate results.

   If the user selected the option to save the results to a report file, an output file selection dialog window will now appear. **ELISA** *for Windows* will suggest *file*.RPT, where *file* is the root name of the standards data file. If this file already exists, it will be appended with information from the current session. If the user elected to analyze an entire plate or file of plates, the output will be stored in this file. If the user selected one unknown, the results will be displayed on screen where they may be stored to file

Figure 19. Dialog window for Module 6 - Calculate Concentrations.

by clicking on the 'Save report to file' option button. If one unknown or one plate was selected for processing, the opening dialog window for this module will reappear after the current round of processing has finished.

If the user selected the option to save the results to a summary file, an output file selection dialog window will appear next. **ELISA** *for Windows* will suggest *file*.SUM, where *file* is the root name of the standards data file. If this file already exists, it will be *updated* with information from the current session. Recording summary results is automatic–it is not necessary to click on a save-to-file button as is the case with the report file. If the user elected to analyze an entire plate or file of plates, the output will be stored in this file. If the user selected one unknown, the results will be displayed on screen and recorded in the summary file. If optical densities are subsequently censored and the unknown recalculated, the summary file will be automatically updated to reflect the revised mean, standard deviation and CV.

Calculation Parameters

ELISA *for Windows* contains a feature that allows users to prescreen optical densities which are included in mean, standard deviation, and CV calculations. This gives an investigator a wide degree of latitude in eliminating observations that would normally be excluded from these calculations without examining each unknown in detail. This is meant as a time-saving device which will facilitate analysis of large numbers of plates in high-volume laboratories. Trying different options and different combinations of options will enable the user to arrive at optimized settings for a given experimental design.

Clicking on the 'SetParams' button will open a new window offering these different options. This window is diagramed in Figure 20.

The 'Set Calculation Parameters' screen offers a number of choices for censoring optical densities. Users may select as many options as desired. A particular optical density will be eliminated from subsequent calculations if it is excluded by any one condition. Essentially, all selected conditions are chained with 'OR' logic.

The first block allows users to enter minimum and maximum OD filters. In this instance, all optical densities below the minimum or above the maximum OD filter settings will be censored.

The next option is a check box which will censor all optical densities which fall outside the range of the standard's optical densities which is listed within parentheses.

The next box allows the user to enter a CV filter. This will effectively exclude from overall mean calculations all ODs for a particular dilution where the within dilution CV is above this selected setting. Enter the number as a percentage, i.e., a number between 1 and 100.

The following box allows users to screen optical densities based on the asymptotes of the fitted standard curve. If the spline fit was selected on the previous screen, this box will be disabled. ODs will be censored if they fall below the lower or above the upper asymptote settings. For reference, the asymptotes are listed in the section title. When a percentage is entered, upon tabbing out of the box or clicking on another option, the program will calculate what the OD filter setting will be and echos this out in the gray area in the right side of the box. For example, if the upper asymptote for the standard curve is 3.5 and the user wishes to include only those unknown ODs that fall within 95% of the upper asymptote of the standard curve fit, then 5.0 would be entered in the 'Include values __ % below the upper asymptote' box. This would set the upper asymptote OD filter at $3.5 - (3.5 \times 0.05) = 3.325$ and all unknown ODs greater than this value will be censored. If the user wishes to use the values of the asymptotes themselves then enter 0.0 in both the lower and upper asymptote text boxes. This is unnecessary, however, as it is impossible to calculate concentrations for ODs that fall outside the range of the asymptotes. The program will flag these situations automatically as non calculable (N/C - see below).

Figure 20. Dialog window for optical density filter settings.

The next box works in the same was the asymptotes box using the range of the standard optical densities in place of the bracketing asymptotes. As with the asymptotes, the values for the minimum and maximum standard OD are listed in the box title. When a percentage is entered, the newly defined filter is calculated and listed in the gray area in the right side of the box. All OD's below the minimum filter or above the maximum filter will be censored.

The final censoring criteria is a check box which instructs the program to exclude *all* optical densities within a specific dilution if any one of them is censored using any one of the other options.

Click on 'Set and Exit' to save the filter settings and apply them to subsequent analyses. They will stay in place until the module is exited to the main menu or they may be erased by clicking on the 'Reset and Exit' button.

Results Window

Figure 21 displays a typical screen displaying the results from this module. A complete listing is provided in Appendix A.

The header comments record the one line title statement if entered by the user in the opening dialog window (Figure 19). Also included are: the file name, the date and time the report was generated, the type of fit, and the four coefficients from the four parameter logistic-log function (which, of course, are missing if the spline fit was chosen in the dialog window, Figure 19). Note that the present parameterization of the logistic-log function determines the lower asymptote of the sigmoidal curve be defined as the **sum** of the first two coefficients. The upper asymptote is equal to the first coefficient. This is all to say that it is impossible to calculate concentrations for optical densities from patient unknowns or quality control samples which fall below the lower asymptote or above the upper asymptote as seen in Figure 22. The range of calculable values is listed in the report just under the coefficients. The report also lists the range of the dilutions for the standard reference serum. Next, the calibration factor is listed. If any optical density filters were set to automatically exclude ODs based on predetermined criteria. They will be detailed next. Each criteria will be followed by a letter in parentheses which is used to annotate the line listing for the report.

The body of the report begins with listing the plate and unknown number selected by the user. It includes a listing of the sample number, and the optical densities which are grouped into blocks of unknowns as specified by the template used to abstract the original data. The dilutions corresponding to the optical densities are also detailed. The average optical density and concentration are given for each group of replicate samples in the unknown series. **ELISA** *for Windows* considers all samples with the same dilution as members of a replicate series. If the unknown did not contain a replicate sample, these averages would simply amount to the single OD and con-

centration. The report also lists the standard deviation and the coefficient of variation (CV) for the replicates. The CV is defined as:

$$(\text{std.dev.}/\text{average concentration}) \times 100.0.$$

If the unknown does not contain a replicate sample the standard deviation and coefficient of variation are set equal to 0.0.

Referring to Figure 21, the 'N/C' designation in the right margin beside the second sample stands for **N**on **C**alculable. The optical densities for sample 2 is outside the range of permissible ODs as detailed in the header comments to the report. The descriptive statistics for the dilution containing this sample has been adjusted to reflect the absence of one calculated concentration value. If a plate is formatted that
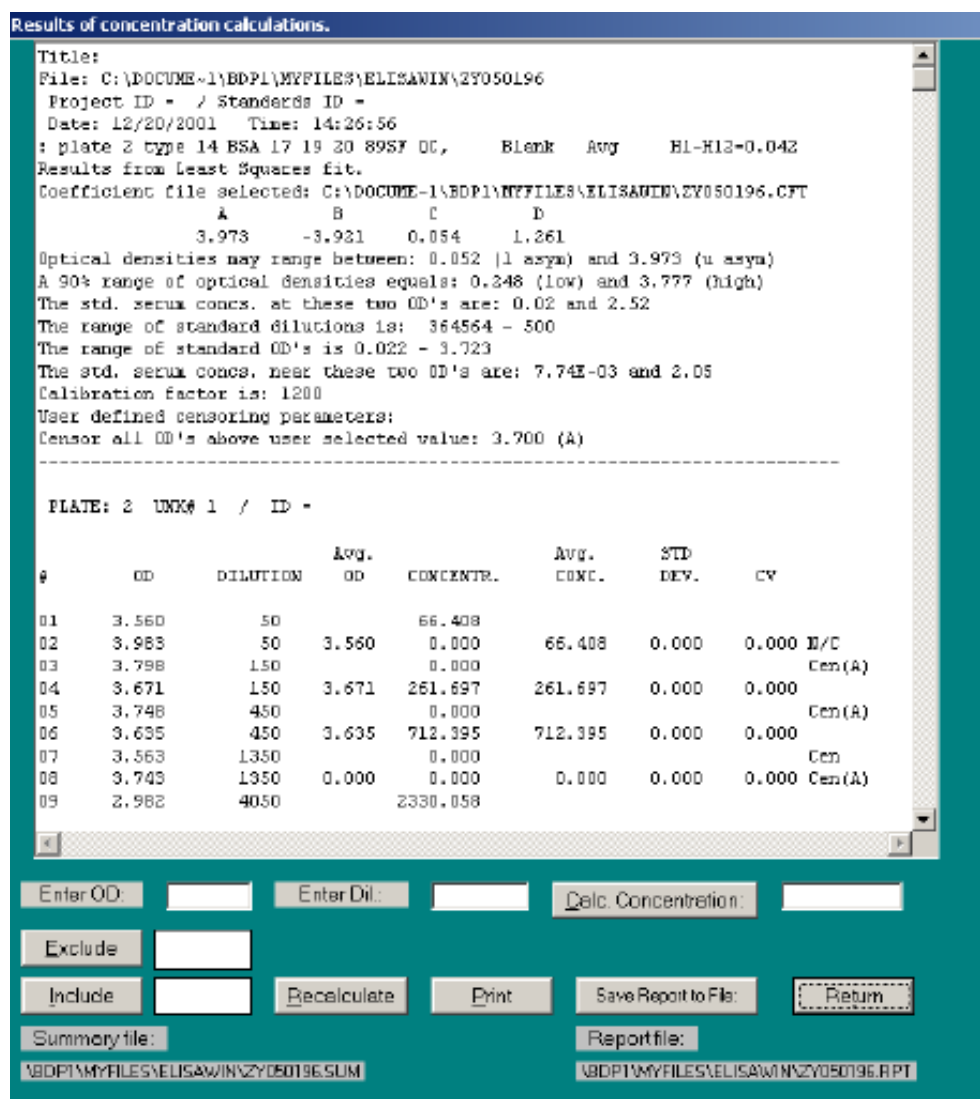
Figure 21. Sample output from Module 6 - Calculate Concentrations.

includes several replicates and only one or two ODs fell out of the range of permissible values, these statistics would still be calculated, using the subset of acceptable optical densities. Sample number 1 and 2 illustrate this, where samples 2 was excluded from the calculations. The average concentration for the pair is simply the value for the remaining sample in the replicate series. Figure 22 illustrates the primary cause for N/C conditions - the observed optical densities fall above or below the upper or lower, respectively, asymptotes for the standard curve.

Samples 3, 5, and 8 are excluded from the summary calculations due to a user defined filtering criteria–their optical densities fell above the preset level of 3.700. The last line lists the grand average concentration, standard deviation, and CV over all calculable samples within the unknown series. Refer to page 57 for a sample of a complete listing.

Typically, the window used to display the results will not be large enough to frame the entire listing. There are horizontal and vertical scroll bars on the bottom and right side of the window, respectively. These scroll bars work the same way as the file list box described in the file selections section of the Introduction. Clicking the left mouse key on the down arrow of the vertical scroll bar will advance the listing one line. Clicking on the bar just above the arrow key will advance the listing by one screen. The horizontal scroll bar is used in the same manner to shift the listing left and right.

It should be noted that users may edit the contents of the output window. Comments may be added to further annotate the output. Several rudimentary Microsoft Word operations will work in these windows. For example, text may be marked by clicking the left mouse key and dragging the cursor through the target text or pressing the shift key while moving the cursor through the text with the cursor control keys. Once text is marked it may be deleted, moved, etc.
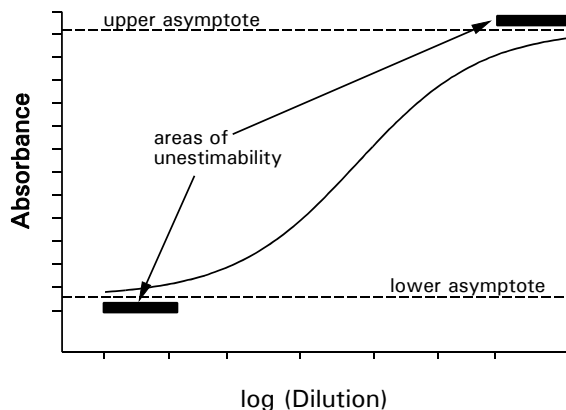
Figure 22. Logistic-log plot showing areas of antibody concentration unestimability.

The options available to the user at this stage appear at the bottom of the output window, beneath the horizontal scroll bar. If the user elected to save the results to a file, the option to save the results will be active and the output file name will be listed underneath the option button. Results will not be saved unless this button is clicked. This allows the user to censor optical densities at will, using many steps and examining intervening results before deciding which set to actually save to the results file. If the user did not select the save file option in the opening dialog window for this module, the 'Save Report to File' option in the output screen will be suppressed. The user may send the contents of the output window directly to the printer. In either case, all additional comments added to the output window will be included during the save and print operations. If the user elected to create a summary file, this will be indicated on the results screen. Results will be recorded automatically when the program returns to the previous screen (clicking on the Return button). In this case the last round of calculations represents the results which will be recorded. If the summary file already contains an entry for the particular plate and unknown, it will be over-written by the present results. This is contrary to the results file where all results are appended to the end of an existing file. Previous results are never over-written.

The first line of options under the results window provides the flexibility of manually calculating antibody concentrations for optical densities not included in the unknowns file. After the optical density and dilution are entered in their respective boxes, clicking the Calc Concentration button will yield the concentration. Dilutions may be entered as the actual decimal number or the reciprocal equivalent (e.g., 0.0033333, 3.333E-3, or 300).

Another option gives the user the ability to include and/or exclude one or more data points and recalculate replicate and overall means, standard deviations, and CVs. This option is displayed as:

<Exclude:>   [   ]
<Include:>   [   ]

The user may enter a single sample number, several numbers or a range of numbers within the range of samples for the unknown series (in the present example, this would be 1-14, listed in the first column of the output window). Single numbers must be separated with a comma (,); a range of numbers is indicated by using a hyphen (-); spaces are optional. Thus

1, 5, 7, 9 - 11, 15

would indicate the operation involves samples 1, 5, 7, 9, 10, 11, and 15.

Once a series of numbers has been entered, clicking on Exclude or Include will mark those sample numbers for exclusion or inclusion from subsequent calculations.

'Ex' and 'In' will appear between the sample number and the optical density to remind the user which sample numbers are to be excluded and included, respectively, from the next round of calculations. This operation may be repeated until just the desired samples are finally included in the summary statistics. To effect these final calculations, click the left mouse button on the Recalculate button. This will revise the output window, excluding/ including all previously marked samples. The notation 'Cen' will appear in the extreme right margin, indicating all samples that have been censured. Excluded samples may be re-included using the Include box. Note that the 'Ex' and 'In' markers will appear temporarily, that is, they disappear after recalculation. Lastly, if the user attempts to include samples that have been automatically censured because they are out of range of permissible optical densities (N/C samples) , **ELISA** *for Windows* will ignore these and continue to exclude them. In this case, the 'N/C' notation will remain in the right margin. Optical densities that were censored due to preset filters may be re-included with the Include function. They will re-enter the summary statistics after clicking on Recalculate.

Additional user comments added to the output window will be lost after recalculation.

Figure 21 displays the result of censuring observation number 7. Again, this technique works in a toggle fashion. That is, the user may experiment with deleting and re-entering specimens to observe the effect on the calculations of the descriptive statistics. If the user desired to reclaim sample number 7, simply entering 7 in the Include box, clicking on Include and then Recalculate would include the sample back into the calculations of means, standard deviations and CVs upon recalculation. Samples 3, 5, and 8 were censured due to preset filtering criteria–their optical densities fell above 3.700. They may also be re-included in the summary calculations using the Include function.

Users must now experiment with these different features to achieve the final desired results. Once the selected samples have been censured, the user may record the results by saving them to a file or printing them. In this fashion, users may record their results at any point in the analysis stage. Alternatively, users may record results at the start of the process to retain a record of the unknown before any data were censured, and then record the output again after all relevant points have been deleted from statistics calculations.

If, in the opening dialog window, the user selects both unknown and quality control samples to be included in the report, they will both appear in the output window with the QC report following the unknown listing (see page 57 for a sample listing). In this case, each will have its own set of descriptive statistics. While the preset filters will operate on both types of samples, the user will only be allowed to include/exclude sample numbers from the unknown series using the Recalculate function in the Report window. It will not be possible to manually censor samples in the QC part of the report. If it is necessary to exclude one or more samples from the

QC block, then select only the QC option for the type of calculations in the opening dialog window (Figure 19).

Clicking on Exit will return the program to the initial dialog window (Figure 19).

A sample summary file is displayed in Figure 23. This file is mainly intended for export into other statistical analysis programs. However, it also serves as a useful compressed listing of results for each unknown and quality control sample in a file containing many plates. This file is grouped by plate and contains information pertinent to each plate. Plates are numbered sequentially. The header or comment lines which precede each plate are prefixed with a period (.) In column 1, followed by the plate number. This will help programmers extract these lines from the following data to facilitate data analysis. Each unknown is represented by one line which contains the overall summary statistics. Each quality control sample contains as many lines as there are unique dilutions for each sample. The dilution is listed for the QC samples and is indicated as combined for the unknowns as their results are summarized over all dilutions.

Upon exiting this module **ELISA** *for Windows* returns to the main menu (Figure 4).

```
. 1
. 1 C:\ELISAWIN\ZY050196  11/18/96 18:25:07
. 1 Plate  1 : Title :
. 1 Lbl Comment : plate 1 type 4 BSA 17 19 20 89SF QC,  Blank   Avg
. 1
. 1 Plt  #  Type   ID                 Dil.    Concent.    S.D.       CV.
. 1
    1   1   U  Unk 1              Combined   178.2268    49.196    27.603
    1   2   U  Unk 2              Combined   838.2361    89.733    10.705
    1   3   U  Unk 3              Combined   547.4713    95.769    17.493
    1   4   U  Unk 4              Combined    80.7430    10.877    13.471
    1   4   Q  2nd qc                 1500     2.4182     0.106     4.399
    1   6   Q  2nd qc                 4500     2.2931     0.073     3.189
    1   8   Q  2nd qc                13500     2.1469     0.024     1.120
    1  10   Q  2nd qc                40500     1.9941     0.045     2.233
    1  12   Q  2nd qc               121500     1.7661     0.016     0.910
    1  14   Q  2nd qc               364500     1.3786     0.387    28.072
```

Figure 23. Sample summary (.SUM) file.

MODULE 7

EDIT DATA FILES

This module allows users to edit data files associated with their ELISA plates. These include raw data (.DAT files), standards data (.STD files), unknowns data (.UNK files), quality control sample data (.QC files), and labels files (.LBL). Files up to 64K may be edited in this module. If an attempt is made to edit a file greater than 64K an error will result and the user will be exited from the module. Users may not edit coefficient data (.CFT files) in this module. If, while estimating the coefficients for a logistic-log standard curve, it is determined that one or more of the standards data points are outliers, this module may be used to remove those points and the estimation procedure retried. Data may also be altered to suit the needs of individual investigators. This may occur with raw data files (.DAT files), where small negative optical densities have been recorded. As explained in the raw data section of the Introduction, negative optical densities will adversely effect parameter estimation and must be converted to non 0 positive numbers. The simple editing capabilities of the present module may be used to change these values.

This module is entered from the main menu (Figure 4) and begins by displaying a data file selection dialog window. Once a file has been selected, it will be placed in a text editing window as shown in Figure 24. The cursor may be moved through the window using a mouse or the cursor control keys.

Users must be careful not to create extra lines by accidentally pressing the ENTER key. Also, standards data (.STD), unknowns data (.UNK), and quality control data (.QC) must be separated by '99999'.

The file may be printed to obtain a hard-copy listing of the file. The window may also be exited without saving and edits, permitting the user to back out of the procedure without changing any data. Finally, the user may save the changes made to the selected data file.

If the user desires to save the changed file, a file selection dialog window will reappear. The current file name will be placed in the file name box. Users may change this file name if they wish to save the changes to a different file.

Upon exiting this module, **ELISA** *for Windows* returns to the main menu (Figure 4).

```
┌─────────────────────────────────────────────────────────────────────┬───┬───┐
│ ─           Text Editor: C:\ELISAWIN\ZY050196.STD                    │ ▼ │ ♦ │
├─────────────────────────────────────────────────────────────────────┴───┴───┤
│  File   Edit                                                                 │
├──────────────────────────────────────────────────────────────────────────┬──┤
│ PLATE: 1 "Test Template","Std 1"                                           │▲ │
│ 1.666666667E-03  0.001                                                     │▒ │
│ 1.666666667E-03  0.001                                                     │▒ │
│ 1.666666667E-03  3.763                                                     │  │
│ 8.333333333E-04  0.007                                                     │  │
│ 8.333333333E-04  0.001                                                     │  │
│ 8.333333333E-04  3.751                                                     │  │
│ 4.166666667E-04  0.001                                                     │  │
│ 4.166666667E-04  0.001                                                     │  │
│ 4.166666667E-04  3.518                                                     │  │
│ 2.083333333E-04  0.001                                                     │  │
│ 2.083333333E-04  0.001                                                     │  │
│ 2.083333333E-04  2.513                                                     │  │
│ 1.041666667E-04  0.001                                                     │  │
│ 1.041666667E-04  0.001                                                     │  │
│ 1.041666667E-04  1.325                                                     │  │
│ 5.208333333E-05  0.001                                                     │  │
│ 5.208333333E-05  0.001                                                     │  │
│ 5.208333333E-05  0.574                                                     │  │
│ 2.604166667E-05  0.001                                                     │  │
│ 2.604166667E-05  0.001                                                     │  │
│ 2.604166667E-05  0.217                                                     │  │
│ 1.302083333E-05  0.001                                                     │  │
│ 1.302083333E-05  0.001                                                     │▼ │
├──┬──────────────────────────────────────────────────────────────────────┬──┤
│ ← │                                                                      │ → │
└──┴──────────────────────────────────────────────────────────────────────┴──┘
```
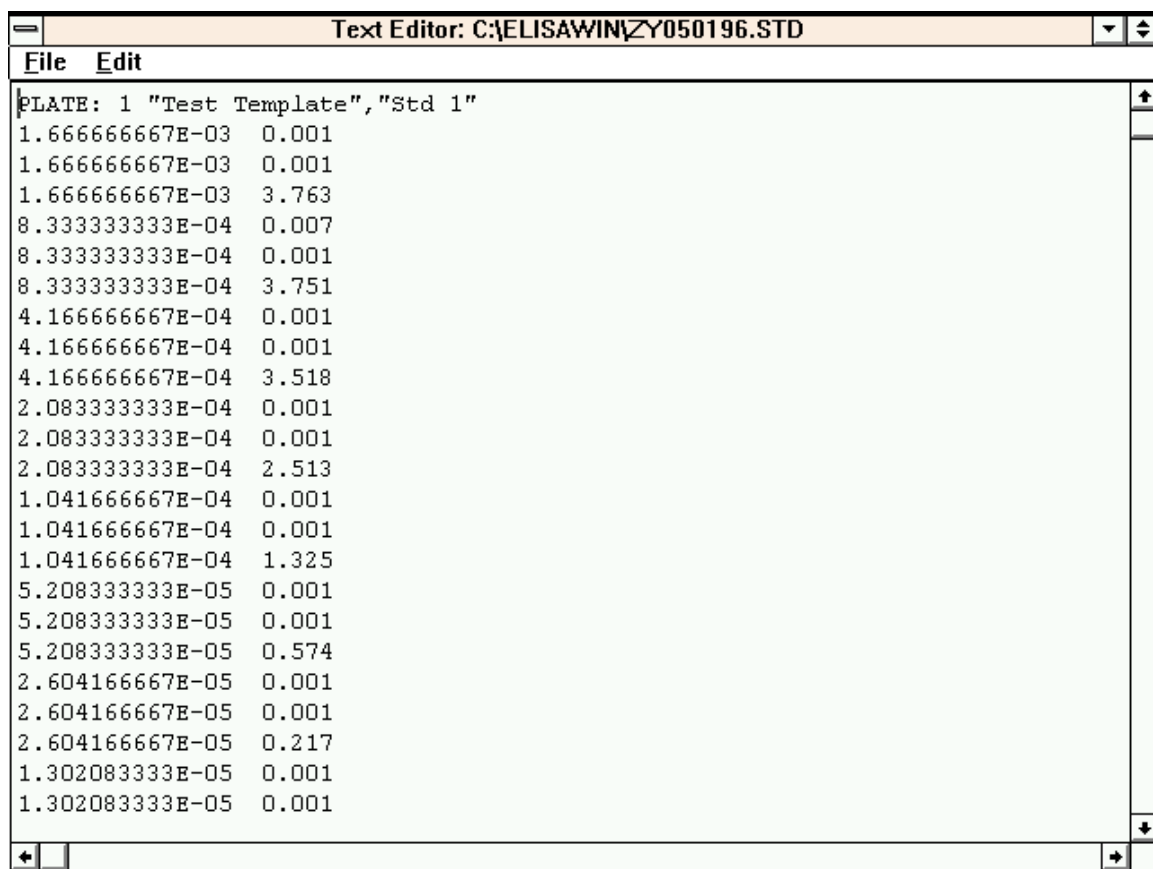
Figure 24. Edit window from Module 7 - Edit Data Files.

APPENDIX A

SAMPLE DATA AND OUTPUT

A collection of sample data files and program output is included on the distribution diskette. These files may be used to test the program and check results after selecting various options in the different modules. The sample data file is MA112190.DAT and appears as:

```
COMMENT:FRASCH ASSAY PB2 1:75 PLATE 1 UNKNOWNS ARE PB2 1:75
  1.716   1.757   1.769   1.840   1.462   0.960   0.562   0.313   0.171   0.090   0.041   1.440
  1.438   1.462   1.458   1.800   1.493   1.013   0.590   0.346   0.173   0.092   0.044   1.409
  0.967   1.014   0.998   1.790   1.484   0.984   0.589   0.334   0.175   0.096   0.047   1.255
  0.586   0.606   0.627   1.801   1.472   0.970   0.588   0.322   0.169   0.085   0.043   1.239
  0.319   0.347   0.346   1.794   1.445   0.958   0.587   0.312   0.175   0.086   0.045   0.233
  0.167   0.186   0.190   1.746   1.424   0.948   0.573   0.312   0.168   0.090   0.046   0.255
  0.094   0.103   0.099   1.791   1.531   0.955   0.584   0.325   0.170   0.091   0.040   0.001
  0.047   0.051   0.051   1.822   1.478   0.906   0.536   0.305   0.158   0.082   0.040   0.001
COMMENT:FRASCH ASSAY PB2 1:75 PLATE 2 UNKNOWNS PB2 1:75
  1.830   1.843   1.914   1.898   1.637   1.045   0.620   0.341   0.178   0.091   0.046   1.446
  1.655   1.681   1.690   1.884   1.628   1.077   0.630   0.350   0.184   0.091   0.047   1.461
  1.078   1.103   1.059   1.896   1.652   1.094   0.641   0.343   0.182   0.099   0.050   1.207
  0.655   0.658   0.635   1.898   1.644   1.091   0.623   0.348   0.173   0.095   0.048   1.203
  0.363   0.361   0.353   1.884   1.653   1.054   0.626   0.352   0.186   0.099   0.050   0.223
  0.193   0.198   0.188   1.914   1.652   1.084   0.624   0.338   0.175   0.090   0.043   0.225
  0.091   0.095   0.094   1.841   1.612   1.079   0.624   0.349   0.172   0.068   0.046   0.001
  0.048   0.047   0.045   1.769   1.603   1.053   0.643   0.326   0.179   0.093   0.046   0.001
```

The template used to abstract the data is located in file FINAL.TPL. Module 2 may be used to examine the template format and the dilution schedules (refer to Module 2 and Appendix B). Users may run the data abstraction module (Module 3) to produce the .STD, .UNK, .QC, and .LBL files. These are also included on the distribution diskette: MA112190.STD, MA112190.UNK, MA112190.QC, and MA112190.LBL.

   Modules 4 may then be used to estimate the four parameters of the logistic-log fit. A sample file of standard curve fitting information is MA112190.FIT contains these results. A portion of that file is reproduced here:

```
File = C:\ELISAWIN\DOWNLOAD\MA112190.STD- Plate: 1
 Project ID = Test Template / Standards ID = Std 1
 Date: 11/19/96   Time: 11:22:53
:FRASCH ASSAY PB2 1:75 PLATE 1 UNKNOWNS ARE PB2 1:75
Results of approximate least squares fit
The procedure used 7 iterations.

Starting values for the iterative process:
     2.28081    -2.32942     .03409     1.00000

                 Rel.      Known    Pred.
        Dil.     Dil.      O.D.     O.D.     Residual   % Error    Weight

   0.001666667   100.00    1.716    1.7559    -0.040     2.325     1.0000
   0.001666667   100.00    1.757    1.7559     0.001     0.063     1.0000
   0.001666667   100.00    1.769    1.7559     0.013     0.741     1.0000
   0.000833333    50.00    1.438    1.4315     0.007     0.453     1.0000
   0.000833333    50.00    1.462    1.4315     0.031     2.087     1.0000
   0.000833333    50.00    1.458    1.4315     0.027     1.818     1.0000
   0.000416667    25.00    0.967    1.0095    -0.042     4.391     1.0000
   0.000416667    25.00    1.014    1.0095     0.005     0.448     1.0000
   0.000416667    25.00    0.998    1.0095    -0.011     1.148     1.0000
   0.000208333    12.50    0.586    0.6097    -0.024     4.047     1.0000
   0.000208333    12.50    0.606    0.6097    -0.004     0.613     1.0000
   0.000208333    12.50    0.627    0.6097     0.017     2.757     1.0000
   0.000104167     6.25    0.319    0.3300    -0.011     3.458     1.0000
   0.000104167     6.25    0.347    0.3300     0.017     4.890     1.0000
   0.000104167     6.25    0.346    0.3300     0.016     4.615     1.0000
   0.000052083     3.12    0.167    0.1732    -0.006     3.697     1.0000
   0.000052083     3.12    0.186    0.1732     0.013     6.896     1.0000
   0.000052083     3.12    0.190    0.1732     0.017     8.856     1.0000
   0.000026042     1.56    0.094    0.0959    -0.002     2.049     1.0000
   0.000026042     1.56    0.103    0.0959     0.007     6.868     1.0000
   0.000026042     1.56    0.099    0.0959     0.003     3.105     1.0000
   0.000013021     0.78    0.047    0.0603    -0.013    28.335     1.0000
   0.000013021     0.78    0.051    0.0603    -0.009    18.270     1.0000
   0.000013021     0.78    0.051    0.0603    -0.009    18.270     1.0000

  The final coefficients (a, b, c, d) are:
     2.13195    -2.10001     0.01841     1.19810

Variances of coefficients = 0.00245     0.00328     0.00000     0.00211

Regular and adjusted sums of squares = 0.007834     0.007834

Approximate multiple R^2 = 0.9991
```

Logistic-log and spline fit plots may be viewed using Module 5.

Module 6 may then be entered to calculate patient unknown antibody concentrations. In this sample data set a calibration factor of 1500 was chosen. A complete listing of these results using the unweighted least squares fit is included in file MA112190.RPT. A sample of the report file is reproduced here:

```
Title:
File: C:\ELISAWIN\DOWNLOAD\MA112190
 Project ID = Test Template / Standards ID = Std 1
 Date: 11/19/96   Time: 11:23:32
:FRASCH ASSAY PB2 1:75 PLATE 1 UNKNOWNS ARE PB2 1:75
Results from Least Squares fit.
Coefficient file selected: C:\ELISAWIN\DOWNLOAD\MA112190.CFT
              A        B        C        D
            2.132    -2.100    0.018    1.198
Optical densities may range between: 0.032 (l asym) and 2.132 (u asym)
The range of standard dilutions is:  76800 - 600
The range of standard OD's is 0.047 - 1.769
Calibration factor is: 1500
--------------------------------------------------------------------------

  PLATE: 1  UNK# 1  /  ID = Unk 1

                           Avg.              Avg.       STD
 #      OD      DILUTION    OD    CONCENTR.   CONC.     DEV.       CV

01    1.840       300            964.043
02    1.800       300    1.820   850.047    907.045    80.607     8.887
03    1.462       600            792.541
04    1.493       600    1.477   839.400    815.970    33.134     4.061
05    0.960      1200            692.807
06    1.013      1200    0.987   754.256    723.532    43.451     6.005
07    0.562      2400            680.189
08    0.590      2400    0.576   720.796    700.493    28.713     4.099
09    0.313      4800            708.477
10    0.346      4800    0.329   789.232    748.855    57.102     7.625
11    0.171      9600            739.668
12    0.173      9600    0.172   749.174    744.421     6.722     0.903
13    0.090     19200            689.887
14    0.092     19200    0.091   710.248    700.068    14.398     2.057
15    0.041     38400            286.910
16    0.044     38400    0.043   364.736    325.823    55.031    16.890
--------------------------------------------------------------------------
Mean (Excludes N/C & Cen):        708.276            166.979    23.575


  PLATE: 1   QUALITY CONTROL

                           Avg.              Avg.       STD
 #      OD      DILUTION    OD    CONCENTR.   CONC.     DEV.       CV

01    1.440      1200            1523.070
02    1.409      1200    1.424   1441.331   1482.200   57.798     3.899
03    1.255      1800            1666.735
04    1.239      1800    1.247   1623.825   1645.280   30.342     1.844
05    0.233     19200            2067.105
06    0.255     19200    0.244   2276.285   2171.695  147.913     6.811
--------------------------------------------------------------------------
Mean (Excludes N/C & Cen):        1766.392           330.317    18.700
```

APPENDIX B

TEMPLATE FILE FORMAT

The FINAL.TPL file which describes a sample template and is distributed with other sample data files along with the program **ELISA** *for Windows* contains the following information:

```
8,"Test Template"
"S"," 1"," 1"," 8"," 3","600","2","L","3","H","Std 1"
"U"," 1"," 4"," 2"," 11","300","2","L","2","V","Unk 1"
"U"," 3"," 4"," 4"," 11","300","2","L","2","V","Unk 2"
"U"," 5"," 4"," 6"," 11","300","2","L","2","V","Unk 3"
"U"," 7"," 4"," 8"," 11","300","2","L","2","V","Unk 4"
"Q"," 1"," 12"," 2"," 12","1200","2","L","2","V","QC1"
"Q"," 3"," 12"," 4"," 12","1800","2","L","2","V","QC2"
"Q"," 5"," 12"," 6"," 12","19200","2","L","2","V","QC3"
```

From Module 2, the format for the 96-well ELISA plate which corresponds to the above file appears as in Figure 25.

Using this illustration, the FINAL.TPL file may be interpreted as follows: The first record contains a single integer which details how many lines follow in the rest of the file. The text to the right of this integer is the Project ID. There are eight lines of information, each of which defines one block of standards, unknowns or QC's.

Each line describes one block and contains 11 fields. These fields are:

1) Block category where S=standards; Q=quality control samples; and U=unknowns.
2) The row number for the top left well in the block.
3) The column number for the top left well in the block.
4) The row number for the bottom right well in the block.
5) The column number for the bottom right well in the block.
6) The starting dilution in the series.
7) The fold change or dilution factor.
8) Indicator for dilution increase or decrease where L=low to high and H=high to low.
9) The number of replicates in the block.
10) "H" for horizontal or "V" for vertical. This details the orientation of the replicates in the block.
11) The block ID.

As an example, the second line of file FINAL.TPL (the first line to follow the number 8) is information pertaining to a standard. The block of wells defining the standard range from row 1, column 1 to row 8, column 3. The initial dilution is 600; the dilution series is 2 fold and the dilutions proceed from low to high. There are three replicates per dilution which run horizontally. The block ID is 'Std 1'.

ELISA Plate Configuration - file -> C:\ELISAWIN\FINAL.TPL

	1	2	3	4	5	6	7	8	9	10	11	12
A	600	600	600	300	600	1200	2400	4800	9600	19200	38400	1200
	S	S	S	U1	U1	U1	U1	U1	U1	U1	U1	QC1
B	1200	1200	1200	300	600	1200	2400	4800	9600	19200	38400	1200
	S	S	S	U1	U1	U1	U1	U1	U1	U1	U1	QC1
C	2400	2400	2400	300	600	1200	2400	4800	9600	19200	38400	1800
	S	S	S	U2	U2	U2	U2	U2	U2	U2	U2	QC2
D	4800	4800	4800	300	600	1200	2400	4800	9600	19200	38400	1800
	S	S	S	U2	U2	U2	U2	U2	U2	U2	U2	QC2
E	9600	9600	9600	300	600	1200	2400	4800	9600	19200	38400	19200
	S	S	S	U3	U3	U3	U3	U3	U3	U3	U3	QC3
F	19200	19200	19200	300	600	1200	2400	4800	9600	19200	38400	19200
	S	S	S	U3	U3	U3	U3	U3	U3	U3	U3	QC3
G	38400	38400	38400	300	600	1200	2400	4800	9600	19200	38400	0
	S	S	S	U4	U4	U4	U4	U4	U4	U4	U4	
H	76800	76800	76800	300	600	1200	2400	4800	9600	19200	38400	0
	S	S	S	U4	U4	U4	U4	U4	U4	U4	U4	

Project ID: Test Template   Print   Return

Figure 25. Display of sample template design.